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(54) Title: GENES INTEGRATING SIGNAL TRANSDUCTION PATHWAYS			
(57) Abstract			
<p>The present invention describes the identification, isolation, sequencing and characterization of two human CalDAG-GEF, and two human cAMP-GEF genes, which are associated with the Ras pathway. Also identified are CalDAG-GEF gene homologues in mice and cAMP-GEF gene homologues in rats. Nucleic acids and proteins comprising or derived from the CalDAG-GEFs and/or cAMP-GEFs are useful in screening and diagnosing certain Ras-associated cancers, in identifying and developing therapeutics for treatment of certain Ras-associated cancers, and in producing cell lines and transgenic animals useful as models of Ras-associated cancers.</p>			

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## GENES INTEGRATING SIGNAL TRANSDUCTION PATHWAYS

### Related Applications

This application claims the benefit of U.S. Application Nos. 60/105,507, filed on October 23, 1998, and 60/108,685, filed on November 16, 1998.

### Field of the Invention

The present invention relates generally to novel polynucleotides and proteins encoded by such polynucleotides, along with therapeutic, diagnostic, and research utilities for these polynucleotides and proteins.

### Background of the Invention

Ras proteins are key regulators of growth, differentiation and malignant transformation. In addition, these proteins are implicated in synaptic function and region-specific learning and memory functions in the brain.

As shown schematically in Figure 1, Ras proteins cycle between inactive GDP-complexed and active GTP-complexed states. GTPase-activating proteins (GAPs) inactivate Ras proteins by stimulating hydrolysis of the bound GTP to GDP, whereas guanine nucleotide exchange factors (GEFs) activate Ras proteins by stimulating release of GDP and the uptake of GTP. So essential are GEFs to Ras action, that genetic loss of GEF function has similar effects to those induced by loss of the Ras proteins themselves. Loss of GEF function can be circumvented by mutations that constitutively activate the Ras proteins, such as an oncogene mutation, or, in some cases, through loss of GAP activity. Activated Ras proteins, which are localized at the plasma membrane, transmit signals from tyrosine kinases to a cascade of serine/threonine kinases, which delivers the signals to the cell nucleus.

Activation of Ras can result in the activation of the mitogen-activated protein (MAP) kinase (also known as extracellular-signal regulated kinase, or ERK) pathway. For example, a receptor tyrosine kinase is activated by a peptide mitogen such as epidermal growth factor (EGF). The EGF-stimulated receptor undergoes autophosphorylation of specific tyrosine residues in its cytoplasmic domain which creates phosphotyrosyl binding sites for the Src homology 2 (SH2) and/or phosphotyrosyl binding (PTB) domains of certain adapter proteins. The adapter protein becomes autophosphorylated on association with activated receptor tyrosine kinases. The GEF is stably associated with the adapter protein which, upon autophosphorylation, mediates translocation of the GEF to the plasma membrane. The GEF then activates the Ras protein. Activated Ras relays its signal downstream through a cascade of cytoplasmic proteins, including

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Raf-1 serine/threonine kinase. The Ras:Raf association promotes translocation of the normally cytoplasmic Raf protein to the plasma membrane, where subsequent events lead to the activation of its kinase function. Upon activation, Raf phosphorylates and activates two MAP kinases (also known as MEKs). MEKs directly associate with the catalytic domain of Raf-1 and are 5 phosphorylated by Raf. Activated MEKs function as dual-specificity kinases and phosphorylate tandem threonine and tyrosine residues in the MAP kinases to activate them. Once activated, the MAP kinases translocate to the nucleus where they phosphorylate and activate a variety of substrates.

10 Rap proteins, members of the Ras small GTPase superfamily, can inhibit Ras signaling of the Ras/Raf-1(a serine/threonine kinase)/MAP kinase pathway or, through B-Raf, can activate MAP kinase. Rap1 consists of two isoforms, Rap1A and Rap1B, which differ mainly at the C-terminus. Characteristic features of Rap1 are its geranylgeranyl modification at the C-terminus, which is responsible for membrane attachments, and a threonine residue at position 61. In most other GTPases, the corresponding residue is a glutamine. Rap proteins, like Ras proteins, cycle 15 between inactive GDP-complexed and active GTP-complexed states. GEFs are required to activate Rap proteins by stimulating the release of GDP and the uptake of GTP.

20 Constitutive activation of the Ras pathway contributes to malignant transformation. In fact, the Ras gene has been implicated in many human cancers, including lung cancer, breast cancer, colorectal cancer, exocrine pancreatic cancer, and myeloid leukemia. Biological and biochemical studies of Ras action indicate that Ras functions like a G-regulatory protein since Ras must be localized in the plasma membrane and must bind with GTP in order to transform 25 cells. Gibbs et al., 53 MICROBIOL. REV. 171-286 (1989).

Targeting components of the Ras signaling pathways has been proposed as one approach 25 for the development of anti-Ras drugs for cancer treatment. One potential approach for targeting Ras for cancer treatment involves the use of farnesyltransferase inhibitors (FTIs). Inhibition of farnesyl-protein transferase, and thereby of farnesylation of the Ras protein, blocks the ability of Ras to transform normal cells to cancer cells. Certain inhibitors of Ras farnesylation cause an increase in soluble Ras which can act as a dominant negative inhibitor of Ras function. While soluble Ras in cancer cells can become a dominant negative inhibitor, soluble Ras in normal cells 30 would not be an inhibitor. A cytosol-localized and activated form of Ras acts as a dominant negative Ras inhibitor of membrane-bound Ras function. Gibbs et al., 86 PROC. NAT'L ACAD.

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Sci. USA 6630-34 (1989). FTIs block Ras function by preventing its post-translational modification by the farnesyl isoprenoid.

Intervention of Ras signaling at multiple or various points can significantly impact the ability of Ras to cause cellular transformation. Since Ras protein function is believed to be crucial to so many cellular processes, targeting only a subset of Ras functions by downstream intervention may provide significant advantages. Thus, there remains a need for identifying additional means for disrupting the Ras pathway. Applicants have discovered four new targets, namely GEFs specific for Rap1A , for disrupting the Ras pathway.

#### Summary of the Invention

Applicants have discovered four mammalian genes which have been designated CalDAG-GEFI, CalDAG-GEFII, cAMP-GEFI, and cAMP-GEFII, which encode proteins having a substrate specificity for Rap1A. The proteins encoded by CalDAG-GEFI and CalDAG-GEFII, referred to herein generally as "CalDAG-GEF," have dual binding domains for calcium and diacylglycerol. The proteins cAMP-GEFI and cAMP-GEFII, referred to herein generally as "cAMP-GEF," have a binding domain for cyclic adenosine 3', 5'-monophosphate. The present disclosure provides polypeptide and polynucleotide sequences for Mus musculus CalDAG-GEFI, Homo sapiens CalDAG-GEFI, Rattus norvegicus CalDAG-GEFII, Homo sapiens CalDAG-GEFII, Rattus norvegicus cAMP-GEFI, Homo sapiens cAMP-GEFI, Homo sapiens alternatively spliced cAMP-GEFI, Rattus norvegicus cAMP-GEFII, and Homo sapiens cAMP-GEFII. See Kawasaki et al., 95 Proc. Natl. Acad. Sci. USA 13278-83 (1998), and Kawasaki et al., 282 Sci. 2275-79 (1998), the disclosures of both of which are incorporated by reference herein.

Thus, in one series of embodiments, the present invention provides isolated nucleic acids including nucleotide sequences comprising or derived from CalDAG-GEF or cAMP-GEF, or encoding polypeptides comprising or derived from CalDAG-GEF or cAMP-GEF proteins. The sequences of the invention include the specifically disclosed sequences, splice variants of these sequences, allelic variants of these sequences, synonymous sequences, and homologous or orthologous variants of these sequences. Thus, for example, the invention provides nucleic acid sequences from the Mus musculus CalDAG-GEFI, Homo sapiens CalDAG-GEFI, Rattus norvegicus CalDAG-GEFII, Homo sapiens CalDAG-GEFII, Rattus norvegicus cAMP-GEFI, Homo sapiens cAMP-GEFI, Homo sapiens alternatively spliced cAMP-GEFI, Rattus norvegicus cAMP-GEFII, and Homo sapiens cAMP-GEFII. The present invention also provides allelic variants and homologous or orthologous sequences by providing methods by which such variants

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may be routinely obtained. Because the nucleic acids of the invention may be used in a variety of diagnostic, therapeutic and recombinant applications, various subsets of the CalDAG-GEF and cAMP-GEF sequences are also provided. For example, for use in allele specific hybridization screening or PCR amplification techniques, subsets of the CalDAG-GEF and cAMP-GEF sequences, including both sense and antisense sequences, and both normal and mutant sequences, as well as intronic, exonic and untranslated sequences, are provided. Such sequences may comprise a small number of consecutive nucleotides from the sequences which are disclosed or otherwise enabled herein, but preferably include at least 8-10, more preferably 10-15, and most preferably 15-25, consecutive nucleotides from a CalDAG-GEF or cAMP-GEF sequence. In another embodiment, such sequences include at least 25-500 consecutive nucleotides from CalDAG-GEF or cAMP-GEF sequence. Other preferred subsets of a CalDAG-GEF or cAMP-GEF sequence include those encoding one or more of the functional domains or antigenic determinants of the CalDAG-GEF or cAMP-GEF protein and, in particular, may include either normal (wild-type) or mutant sequences. The invention also provides for various nucleic acid constructs in which CalDAG-GEF or cAMP-GEF sequences, either complete or subsets, are operably joined to exogenous sequences to form cloning vectors, expression vectors, fusion vectors, transgenic constructs, and the like. Thus, in accordance with another aspect of the invention, a recombinant vector for transforming a mammalian or invertebrate tissue cell to express a normal or mutant CalDAG-GEF and/or cAMP-GEF sequence in the cells is provided.

In another series of embodiments, the present invention provides for host cells which have been transfected or otherwise transformed with one of the nucleic acids of the invention. The cells may be transformed merely for purposes of propagating the nucleic acid constructs of the invention, or may be transformed so as to express the CalDAG-GEF and/or cAMP-GEF sequences. The transformed cells of the invention may be used in assays to identify proteins and/or other compounds which affect normal or mutant CalDAG-GEF and/or cAMP-GEF expression, which interact with the normal or mutant CalDAG-GEF and/or cAMP-GEF proteins, and/or which modulate the function or effects of the normal or mutant proteins, or to produce the CalDAG-GEF and/or cAMP-GEF proteins, fusion proteins, functional domains, antigenic determinants, and/or antibodies of the invention. Transformed cells may also be implanted into hosts, including humans, for therapeutic or other reasons. Preferred host cells include mammalian cells, including pure or mixed cell cultures, as well as bacterial, yeast, nematode,

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insect and other invertebrate cells. For uses as described below, preferred cells also include embryonic stem cells, zygotes, gametes, and germ line cells.

In another series of embodiments, the present invention provides transgenic animal models of diseases or disorders associated with mutations in the CalDAG-GEF and/or cAMP-GEF genes. The animal may be essentially any non-human mammal, including rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates. In addition, invertebrate models, including nematodes and insects, may be used for certain applications. The animal models are produced by standard transgenic methods including microinjection, electroporation, transfection, or other forms of transformation of embryonic stem cells, zygotes, gametes, and germ line cells with vectors including genomic or cDNA fragments, minigenes, homologous recombination vectors, viral insertion vectors and the like. Suitable vectors include vaccinia virus, adenovirus, adeno-associated virus, retrovirus, liposome transport, neuraltropic viruses, and Herpes simplex virus. The animal models may include transgenic sequences comprising or derived from the CalDAG-GEF and/or cAMP-GEF genes, including normal and mutant sequences, intronic, exonic and untranslated sequences, and sequences encoding subsets of the CalDAG-GEF and/or cAMP-GEF proteins, such as functional domains. The major types of animal models provided include: (1) Animals in which a normal human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a recombinant gene or a large genomic fragment; in which a normal human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly substituted for one or both copies of the animal's homologous CalDAG-GEF and/or cAMP-GEF gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's homologous CalDAG-GEF and/or cAMP-GEF genes have been recombinantly "humanized" by the partial substitution of sequences encoding the human homologue by homologous recombination or gene targeting; (2) Animals in which a mutant human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a recombinant gene or a large genomic fragment; in which a mutant human CalDAG-GEF and/or cAMP-GEF gene has been recombinantly substituted for one or both copies of the animal's homologous CalDAG-GEF and/or cAMP-GEF gene by homologous recombination or gene targeting; and/or in which one or both copies of one of the animal's

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homologous CalDAG-GEF and/or cAMP-GEF gene have been recombinantly "humanized" by the partial substitution of sequences encoding a mutant human homologue by homologous recombination or gene targeting; (3) Animals in which a mutant version of one of that animal's CalDAG-GEF or cAMP-GEF gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a recombinant gene or a large genomic fragment; and/or in which a mutant version of one of that animal's CalDAG-GEF or cAMP-GEF gene has been recombinantly substituted for one or both copies of the animal's homologous CalDAG-GEF or cAMP-GEF gene by homologous recombination or gene targeting; and (4) "Knock-out" animals in which one or both copies of one of the animal's CalDAG-GEF or cAMP-GEF genes have been partially or completely deleted by homologous recombination or gene targeting, or have been inactivated by the insertion or substitution by homologous recombination or gene targeting of exogenous sequences.

In another series of embodiments, the present invention provides for substantially pure protein preparations including polypeptides comprising or derived from the CalDAG-GEF and/or cAMP-GEF proteins. The CalDAG-GEF and cAMP-GEF protein sequences of the invention include the specifically disclosed sequences, variants of these sequences resulting from alternative mRNA splicing, allelic variants of these sequences, and homologous or orthologous variants of these sequences. Thus, for example, the invention provides amino acid sequences from the *Mus musculus* CalDAG-GEFI protein, *Homo sapiens* CalDAG-GEFI protein, *Rattus norvegicus* CalDAG-GEFII protein, *Homo sapiens* CalDAG-GEFII protein, *Rattus norvegicus* cAMP-GEFI protein, *Homo sapiens* cAMP-GEFI protein, *Homo sapiens* alternatively spliced cAMP-GEFI protein, *Rattus norvegicus* cAMP-GEFII protein, and *Homo sapiens* cAMP-GEFII protein. The present invention also provides allelic variants and homologous or orthologous proteins by providing methods by which such variants may be routinely obtained. The present invention also specifically provides for mutant or disease-causing variants of CalDAG-GEF and cAMP-GEF by providing methods by which such variants may be routinely obtained. Because the proteins of the invention may be used in a variety of diagnostic, therapeutic and recombinant applications, various subsets of the CalDAG-GEF and cAMP-GEF protein sequences and combinations of the CalDAG-GEF and cAMP-GEF protein sequences with heterologous sequences are also provided. For example, for use as immunogens or in binding assays, subsets of the CalDAG-GEF and cAMP-GEF protein sequences, including both normal and mutant

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sequences, are provided. Such protein sequences may comprise a small number of consecutive amino acid residues from the sequences which are disclosed or otherwise enabled herein, but preferably include at least 4-8, and preferably at least 9-15 consecutive amino acid residues from a CalDAG-GEF or cAMP-GEF sequence. In another embodiment, such sequences comprise at 5 least 15-200 consecutive amino acid residues from a CalDAG-GEF or cAMP-GEF sequence. Other preferred subsets of the CalDAG-GEF and cAMP-GEF protein sequences include those corresponding to one or more of the functional domains or antigenic determinants of the CalDAG-GEF and cAMP-GEF proteins and, in particular, may include either normal (wild-type) or mutant sequences. The invention also provides for various protein constructs in which a 10 CalDAG-GEF and/or cAMP-GEF sequences, either complete or subsets thereof, are joined to exogenous sequences to form fusion proteins and the like. In accordance with these embodiments, the present invention also provides for methods of producing all of the above described proteins which comprise, or are derived from, CalDAG-GEF and/or cAMP-GEF.

In another series of embodiments, the present invention provides for the production and 15 use of polyclonal and monoclonal antibodies, including antibody fragments, including Fab fragments, F(ab')<sub>2</sub>, and single chain antibody fragments, which selectively bind to CalDAG-GEF or cAMP-GEF, or to specific antigenic determinants of CalDAG-GEF or cAMP-GEF. The antibodies may be raised in mouse, rabbit, goat or other suitable animals, or may be produced recombinantly in cultured cells such as hybridoma cell lines. Preferably, the antibodies 20 selectively bind to a sequence comprising at least 4-8, and preferably at least 9-15, consecutive amino acid residues from a CalDAG-GEF or cAMP-GEF sequence. The antibodies of the invention may be used in the various diagnostic, therapeutic and technical applications described herein.

In another series of embodiments, the present invention provides methods of screening or 25 identifying proteins, small molecules or other compounds which are capable of inducing or inhibiting the expression and/or function of the CalDAG-GEF and/or cAMP-GEF genes or proteins. The assays may be performed *in vitro* using non-transformed cells, immortalized cell lines, or recombinant cell lines, or *in vivo* using the transgenic animal models enabled herein. In particular, the assays may detect the presence of increased or decreased expression of CalDAG- 30 GEF and/or cAMP-GEF-related genes or proteins on the basis of increased or decreased mRNA expression, increased or decreased levels of CalDAG-GEF and/or cAMP-GEF-related protein products, or increased or decreased levels of expression of a marker gene (e.g.,  $\beta$ -galactosidase,

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- green fluorescent protein, alkaline phosphatase or luciferase) operably joined to a 5' regulatory region in a recombinant construct. Cells known to express CalDAG-GEF or cAMP-GEF, or transformed to express CalDAG-GEF or cAMP-GEF, are incubated and one or more test compounds are added to the medium. After allowing a sufficient period of time (e.g., 0-72 hours) for the compound to induce or inhibit the expression of the CalDAG-GEF or cAMP-GEF, any change in levels of expression from an established baseline may be detected using any of the techniques described above. In particularly preferred embodiments, the cells are from an immortalized cell line such as a human neuroblastoma, glioblastoma or a hybridoma cell line, or are transformed cells of the invention.
- In another series of embodiments, the present invention provides methods for identifying proteins and other compounds which bind to, or otherwise directly interact with, CalDAG-GEF and/or cAMP-GEF. The proteins and compounds will include endogenous cellular components which interact with the CalDAG-GEF and/or cAMP-GEF *in vivo* and which, therefore, provide new targets for pharmaceutical and therapeutic interventions, as well as recombinant, synthetic, and otherwise exogenous compounds which may have CalDAG-GEF and/or cAMP-GEF binding capacity and, therefore, may be candidates for pharmaceutical agents. Thus, in one series of embodiments, cell lysates or tissue homogenates (e.g., human brain homogenates, lymphocyte lysates) may be screened for proteins or other compounds which bind to one of the normal or mutant CalDAG-GEF or cAMP-GEF proteins. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (e.g., libraries of small molecules or peptides), may be screened for CalDAG-GEF or cAMP-GEF binding capacity. In each of these embodiments, an assay is conducted to detect binding between a "CalDAG-GEF component" or a "cAMP-GEF component" and some other moiety. In one embodiment, a CalDAG-GEF component comprises a CalDAG-GEF SRC1, SRC2, SRC3, EF hand or a DAG-binding domain.
- In another embodiment, a cAMP-GEF component comprises a cAMP-GEF SRC1, SRC2, SRC3, or a cAMP-binding domain. The "CalDAG-GEF component" or the "cAMP-GEF component" in these assays may be any polypeptide comprising or derived from a normal or mutant CalDAG-GEF or cAMP-GEF protein, including functional domains or antigenic determinants of CalDAG-GEF or cAMP-GEF, or CalDAG-GEF or cAMP-GEF fusion proteins. Binding may be detected by non-specific measures (e.g., changes in intracellular Ca<sup>2+</sup>, GTP/GDP ratio) or by specific measures (e.g., changes in the expression of downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential hybridization, or SAGE methods). The

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preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-isolation of CalDAG-GEF or cAMP-GEF components and bound proteins or other compounds by immunoprecipitation; (3) the Biomolecular Interaction Assay (BIAcore); and (4) the yeast two-hybrid systems.

5 In another series of embodiments, the present invention provides for methods of identifying proteins, small molecules and other compounds capable of modulating the activity of normal or mutant CalDAG-GEF or cAMP-GEF. Using normal cells or animals, the transformed cells and transgenic animal models of the present invention, or cells obtained from subjects bearing normal or mutant CalDAG-GEF or cAMP-GEF genes, the present invention provides  
10 methods of identifying such compounds on the basis of their ability to affect the expression of CalDAG-GEF and/or cAMP-GEF, the intracellular localization of the CalDAG-GEF and/or cAMP-GEF, or other biochemical, histological, or physiological markers which distinguish cells bearing normal and mutant CalDAG-GEF and/or cAMP-GEF sequences. Using the transgenic animals of the invention, methods of identifying such compounds are also provided on the basis  
15 of the ability of the compounds to affect behavioral, physiological or histological phenotypes associated with mutations in CalDAG-GEF and/or cAMP-GEF.

In another series of embodiments, the present invention provides methods and reagents for the screening and diagnosis of diseases or disorders associated with mutations in the CalDAG-GEF and/or cAMP-GEF genes. Screening and/or diagnosis can be accomplished by  
20 methods based upon the nucleic acids (including genomic and mRNA/cDNA sequences), proteins, and/or antibodies disclosed and enabled herein, including functional assays designed to detect failure or augmentation of the normal CalDAG-GEF and/or cAMP-GEF activity and/or the presence of specific new activities conferred by the mutant CalDAG-GEF and/or cAMP-GEF. Thus, for example, screens and diagnostics based upon CalDAG-GEF and/or cAMP-GEF  
25 proteins are provided which detect differences between mutant and normal CalDAG-GEF or cAMP-GEF in electrophoretic mobility, in proteolytic cleavage patterns, in molar ratios of the various amino acid residues, or in ability to bind specific antibodies. In addition, screens and diagnostics based upon nucleic acids (gDNA, cDNA or mRNA) are provided which detect differences in nucleotide sequences by direct nucleotide sequencing, hybridization using allele  
30 specific oligonucleotides, restriction enzyme digest and mapping (e.g., RFLP, REF-SSCP), electrophoretic mobility (e.g., SSCP, DGGE), PCR mapping, RNase protection, chemical mismatch cleavage, ligase-mediated detection, and various other methods. Other methods are

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- also provided which detect abnormal processing of CalDAG-GEF and/or cAMP-GEF or proteins reacting with CalDAG-GEF and/or cAMP-GEF, alterations in CalDAG-GEF and/or cAMP-GEF transcription, translation, and post-translational modification; alterations in the intracellular and extracellular trafficking of CalDAG-GEF and/or cAMP-GEF gene products; or abnormal
- 5 intracellular localization of CalDAG-GEF and/or cAMP-GEF. Such methods and reagents are also useful in the analysis of neoplasias and mammalian immune system function, as well as functional *in vivo* imaging of mammalian organ systems. In accordance with these embodiments, diagnostic kits are also provided which will include the reagents necessary for the above-described diagnostic screens.
- 10 In another series of embodiments, the present invention provides methods and therapeutic agents for use in the treatment of conditions such as neurological and neuropsychiatric disorders such as Huntington's disease, Parkinson's disease, Alzheimer's disease, dystonia, Tourette's syndrome, obsessive-compulsive disorder, attention deficit/hyperactive disorder, depression, schizophrenia, and stroke; neoplasias such as solid tumors including colon, breast, lung, prostate,
- 15 and hematopoietic tumors such as leukemia, Hodgkins, and non-Hodgkins lymphomas; and autoimmune diseases, allergies, and asthma; as well as for the enhancement of the immune response in normal and immunocompromised individuals. These methods and therapeutic agents may be based upon (1) administration of normal CalDAG-GEF and/or cAMP-GEF proteins; (2) gene therapy with normal CalDAG-GEF and/or cAMP-GEF genes to compensate for or replace
- 20 the mutant genes; (3) gene therapy based upon antisense sequences to mutant CalDAG-GEF and/or cAMP-GEF genes or upon sequences which "knock-out" the mutant genes; (4) gene therapy based upon sequences which encode a protein which blocks or corrects the deleterious effects of CalDAG-GEF and/or cAMP-GEF mutants; (5) immunotherapy based upon antibodies to normal and/or mutant CalDAG-GEF and/or cAMP-GEF proteins; or (6) small molecules
- 25 (drugs) which alter CalDAG-GEF and/or cAMP-GEF expression, block interactions between (normal or mutant) forms of CalDAG-GEF and/or cAMP-GEF and other proteins or ligands, or which otherwise block the function of (normal or mutant) CalDAG-GEF and/or cAMP-GEF proteins by altering the structure of the proteins, by enhancing their metabolic clearance, or by inhibiting their function.
- 30 In accordance with another aspect of the invention, the proteins of the invention can be used as starting points for rational drug design to provide ligands, therapeutic drugs or other types of small chemical molecules. Alternatively, small molecules or other compounds

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identified by the above-described screening assays may serve as "lead compounds" in rational drug design.

#### Brief Description of the Drawings

Figure 1 is a partial schematic diagram of a Ras pathway.

5       Figure 2A shows human (h) and mouse (m) CalDAG-GEFI, human (h) and rat (r) CalDAG-GEFII, and *C. elegans* (cel) (F25B3.3, GenBank accession number: 1262950) CalDAG-GEF. Figure 2B shows a computer-generated phylogenetic tree analysis of the GEF domains of CalDAG-GEFI and CalDAG-GEFII in relation to other Ras-superfamily GEFs. Figure 2C shows multiple alignment of GEF structurally conserved regions (SCRs) of CalDAG-  
10      GEFs and several other GEFs of the Ras superfamily. Figure 2D shows the full-length amino acid sequences of human (h) and mouse (m) CalDAG-GEFI (box indicates amino acid differences). Figure 2E shows the sequence similarity (black indicates identity) of EF-hand domains in CalDAG-GEFs and other calcium binding proteins. Figure 2F shows the sequence similarity of DAG-binding domains of CalDAG-GEFs and PKC (protein kinase C) family  
15      proteins.

Figure 3A is a schematic representation of cAMP-GEF family proteins, including human (h) and rat (r) cAMP-GEFI, human (h) cAMP-GEFII and *C. elegans* (cel) (T2OG5.5, GenBank accession number: 458480) cAMP-GEF. Figure 3B is a phylogenetic tree analysis of cAMP binding domains of cAMP-GEFI and II and other cyclic nucleotide binding proteins. Figure 3C is a phylogenetic tree analysis of GEF domains of cAMP-GEFI and II and other Ras superfamily GEFs. Figure 3D shows the amino acid sequences of the structurally conserved regions (SCRs) of cAMP-GEFs and other Ras superfamily GEFs (black indicates identity). Figure 3E shows the amino acid sequences of the cAMP binding pockets of cAMP-GEFI and II and other cyclic nucleotide-binding proteins. The positions of invariant amino acid residues are shown by black diamonds. The open diamond indicates the amino acid that determines the binding specificity for cAMP or cGMP. The arrow indicates the position of amino acid substitutions specific to cAMP-GEFs. Figure 3F is the full-length amino acid sequences of human cAMP-GEFI and II (boxes indicate amino acid identity).

#### Detailed Description of the Invention

30       The present invention is based, in part, upon the discovery of a family of mammalian genes which are associated with the Ras pathway. The discovery of these genes, designated

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CalDAG-GEFI, CalDAG-GEFII, cAMP-GEFI, and cAMP-GEFII, as well as the characterization of these genes, their protein products, mutants, and possible functional roles, are described below.

### I. Definitions

5 In order to facilitate review of the various embodiments of the invention, and an understanding of the various elements and constituents used in making and using the invention, the following definitions are provided for particular terms used in the description and the claims which follow:

10 CalDAG-GEF. As used without further modification herein, the terms "CalDAG-GEF" or "CalDAG-GEFs" refer to the CalDAG-GEFI and/or the CalDAG-GEFII genes/proteins. In particular, the unmodified terms "CalDAG-GEF" or "CalDAG-GEFs" refer to the mammalian genes/proteins and, preferably, the human genes/proteins.

15 cAMP-GEF. As used without further modification herein, the terms "cAMP-GEF" or "cAMP-GEFs" refer to the cAMP-GEFI and/or the cAMP-GEFII genes/proteins. In particular, the unmodified terms "cAMP-GEF" or "cAMP-GEFs" refer to the mammalian genes/proteins and, preferably, the human genes/proteins.

20 CalDAG-GEF gene. As used herein, the term "CalDAG-GEF gene" means the mammalian genes represented by SEQ ID NOS: 1, 3, 5, and 7, as well as any allelic variants and heterospecific mammalian homologues. A murine CalDAG-GEFI cDNA sequence is disclosed herein as SEQ ID NO: 1, and a human CalDAG-GEFI cDNA sequence is disclosed herein as SEQ ID NO: 3. A rat CalDAG-GEFII cDNA sequence is disclosed herein as SEQ ID NO: 5, and a human CalDAG-GEFII cDNA sequence is disclosed herein as SEQ ID NO: 7. The term "CalDAG-GEF gene" primarily relates to a coding sequence, but can also include some or all of the flanking regulatory regions and/or introns. The term "CalDAG-GEF gene" specifically 25 includes artificial or recombinant genes created from cDNA or genomic DNA, including recombinant genes based upon splice variants.

30 CalDAG-GEF protein. As used herein, the term "CalDAG-GEF protein" means a protein encoded by a CalDAG-GEF gene, including allelic variants and heterospecific mammalian homologues. A murine CalDAG-GEFI protein sequence is disclosed herein as SEQ ID NO: 2, and a human CalDAG-GEFI protein sequence is disclosed herein as SEQ ID NO: 4. A rat CalDAG-GEFII protein sequence is disclosed herein as SEQ ID NO: 6, and a human CalDAG-GEFII protein sequence is disclosed herein as SEQ ID NO: 8. Splice variants are also embraced

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by the term CalDAG-GEF protein as used herein. The protein may be produced by recombinant cells or organisms, may be substantially purified from natural tissues or cell lines, or may be synthesized chemically or enzymatically. Therefore, the term "CalDAG-GEF protein" is intended to include the protein in glycosylated, partially glycosylated, or unglycosylated forms, as well as in phosphorylated, partially phosphorylated, unphosphorylated, sulphated, partially sulphated, or unsulphated forms. The term also includes allelic variants and other functional equivalents of the CalDAG-GEF amino acid sequences, including biologically active proteolytic or other fragments.

hCalDAG-GEF gene and/or protein. As used herein, the abbreviation "hCalDAG-GEF" refers to the human homologue and human allelic variants of the CalDAG-GEF genes and/or proteins. Two cDNA sequences of the human CalDAG-GEF genes are disclosed herein as SEQ ID NOS: 3 and 7. The corresponding hCalDAG-GEF protein sequences are disclosed herein as SEQ ID NOS: 4 and 8. Allelic variants, including deleterious mutants, are enabled in the description which follows.

mCalDAG-GEF gene and/or protein. As used herein, the abbreviation "mCalDAG-GEF" refers to the murine homologues and murine allelic variants of the CalDAG-GEF gene and/or protein. A cDNA sequence of one murine CalDAG-GEF gene is disclosed herein as SEQ ID NO: 16. The corresponding mCalDAG-GEF protein sequence is disclosed herein as SEQ ID NO: 17. Allelic variants, including deleterious mutants, are enabled in the description which follows.

rCalDAG-GEF gene and/or protein. As used herein, the abbreviation "rCalDAG-GEF" refers to the rat homologue and rat allelic variants of the CalDAG-GEF genes and/or proteins. A cDNA sequence of one rat CalDAG-GEF gene is disclosed herein as SEQ ID NO: 5. The corresponding rCalDAG-GEF protein sequence is disclosed herein as SEQ ID NO: 6. Allelic variants, including deleterious mutants, are enabled in the description which follows.

cAMP-GEF gene. As used herein, the term "cAMP-GEF gene" means the mammalian genes represented by SEQ ID NOS: 9, 11, 13, 15, and 17, as well as any allelic variants and heterospecific mammalian homologues. A rat cAMP-GEFI cDNA sequence is disclosed herein as SEQ ID NO: 9, and a human cAMP-GEFI cDNA sequence is disclosed as SEQ ID NO: 11. Another human cAMP-GEFI cDNA sequence, resulting from alternative splicing of the mRNA transcript, is disclosed as SEQ ID NO: 13. A rat cAMP-GEFII cDNA sequence is disclosed as SEQ ID NO: 15, and a human cAMP-GEFII cDNA sequence is disclosed as SEQ ID NO: 17.

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The term "cAMP-GEF gene" primarily relates to a coding sequence, but can also include some or all of the flanking regulatory regions and/or introns. The term cAMP-GEF gene specifically includes artificial or recombinant genes created from cDNA or genomic DNA, including recombinant genes based upon splice variants.

- 5        cAMP-GEF protein. As used herein, the term "cAMP-GEF protein" means a protein encoded by a cAMP-GEF gene, including allelic variants and heterospecific mammalian homologues. A rat cAMP-GEFI protein sequence is disclosed herein as SEQ ID NO: 10, and a human cAMP-GEFI protein sequence is disclosed as SEQ ID NO: 12. Another human cAMP-GEFI protein sequence, resulting from alternative splicing of the mRNA transcript, is disclosed  
10      as SEQ ID NO: 14. A rat cAMP-GEFII protein sequence is disclosed as SEQ ID NO: 16, and a human cAMP-GEFII protein sequence is disclosed as SEQ ID NO: 18. Splice variants are also embraced by the term cAMP-GEF protein as used herein. The protein may be produced by recombinant cells or organisms, may be substantially purified from natural tissues or cell lines, or may be synthesized chemically or enzymatically. Therefore, the term "cAMP-GEF protein" is  
15      intended to include the protein in glycosylated, partially glycosylated, or unglycosylated forms, as well as in phosphorylated, partially phosphorylated, unphosphorylated, sulphated, partially sulphated, or unsulphated forms. The term also includes allelic variants and other functional equivalents of the cAMP-GEF amino acid sequences, including biologically active proteolytic or other fragments.  
20        hcAMP-GEF gene and/or protein. As used herein, the abbreviation "hcAMP-GEF" refers to the human homologue and human allelic variants of the cAMP-GEF gene and/or protein. One cDNA sequences of the human cAMP-GEF gene is disclosed herein as SEQ ID NO: 18. The corresponding hcAMP-GEF protein sequence is disclosed herein as SEQ ID NO: 19. Numerous allelic variants, including deleterious mutants, are disclosed and enabled throughout the  
25      description which follows.  
30        rcAMP-GEF gene and/or protein. As used herein, the abbreviation "rcAMP-GEF" refers to the rat homologue and rat allelic variants of the cAMP-GEF gene and/or protein. Two cDNA sequences of rat cAMP-GEF genes are disclosed herein as SEQ ID NOS: 9 and 15. The corresponding rcAMP-GEF protein sequences are disclosed herein as SEQ ID NOS: 10 and 16.. Numerous allelic variants, including deleterious mutants, are disclosed and enabled throughout  
the description which follows.

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Normal. As used herein with respect to genes, the term "normal" refers to a gene which encodes and expresses a normal protein. As used herein with respect to proteins, the term "normal" means a protein which performs its usual or normal physiological role and which is not associated with, or causative of, a pathogenic condition or state. Therefore, as used herein, 5 the term "normal" is essentially synonymous with the usual meaning of the phrase "wild type." For any given gene, or corresponding protein, a multiplicity of normal allelic variants may exist, none of which is associated with the development of a pathogenic condition or state. Such normal allelic variants include, but are not limited to, variants in which one or more nucleotide substitutions do not result in a change in the encoded amino acid sequence.

10        Mutant. As used herein with respect to genes, the term "mutant" refers to a gene which encodes a mutant protein and/or fails to express a normal protein. As used herein with respect to proteins, the term "mutant" means a protein which does not perform its usual or normal physiological role and which is associated with, or causative of, a pathogenic condition or state. Therefore, as used herein, the term "mutant" is essentially synonymous with the terms 15 "dysfunctional," "pathogenic," "disease-causing," and "deleterious." With respect to the CalDAG-GEF and cAMP-GEF genes and proteins of the present invention, the term "mutant" refers to CalDAG-GEF and cAMP-GEF genes/proteins bearing one or more nucleotide/amino acid substitutions, insertions and/or deletions which cause the genes/proteins to be dysfunctional, pathogenic, disease-causing or otherwise deleterious. This definition is understood to include the 20 various mutations that naturally exist, including but not limited to those disclosed herein, as well as synthetic or recombinant mutations produced by human intervention. The term "mutant," as applied to the CalDAG-GEF and cAMP-GEF genes, is not intended to embrace sequence variants which, due to the degeneracy of the genetic code, encode proteins identical to the normal sequences disclosed or otherwise enabled herein; nor is it intended to embrace sequence variants 25 which, although they encode different proteins, encode proteins which are functionally equivalent to normal CalDAG-GEF and/or cAMP-GEF proteins.

30        Functional equivalent. As used herein in describing gene sequences and amino acid sequences, the term "functional equivalent" means that a recited sequence need not be identical to a particularly disclosed sequence of the SEQ ID NOs but need only provide a sequence which functions biologically and/or chemically as the equivalent of the disclosed sequence.

Substantially pure. As used herein with respect to protein preparations, the term "substantially pure" means a preparation which contains at least 60% (by dry weight) the protein

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of interest, exclusive of the weight of other intentionally included compounds. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by dry weight the protein of interest, exclusive of the weight of other intentionally included compounds. Purity can be measured by any appropriate method, e.g., column chromatography, gel electrophoresis, or HPLC analysis. If a preparation intentionally includes two or more different proteins of the invention, a "substantially pure" preparation means a preparation in which the total dry weight of the proteins of the invention is at least 60% of the total dry weight, exclusive of the weight of other intentionally included compounds. Preferably, for such preparations containing two or more proteins of the invention, the total weight of the proteins of the invention be at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total dry weight of the preparation, exclusive of the weight of other intentionally included compounds. Thus, if the proteins of the invention are mixed with one or more other proteins (e.g., serum albumin, 6-OST) or compounds (e.g., diluents, detergents, excipients, salts, polysaccharides, sugars, lipids) for purposes of administration, stability, storage, and the like, the weight of such other proteins or compounds is ignored in the calculation of the purity of the preparation.

Isolated nucleic acid. As used herein, an "isolated nucleic acid" is a ribonucleic acid, deoxyribonucleic acid, or nucleic acid analog comprising a polynucleotide sequence that has been isolated or separated from sequences that are immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant nucleic acid which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences and/or including exogenous regulatory elements.

Transformed cell. As used herein, a "transformed cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid molecule of interest. The nucleic acid of interest will typically encode a peptide or protein. The transformed cell may express the sequence of interest or may be used only to propagate the sequence. The term "transformed" may be used herein to embrace any method of introducing

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exogenous nucleic acids including, but not limited to, transformation, transfection, electroporation, microinjection, viral-mediated transfection, and the like.

Operably joined. As used herein, a coding sequence and a regulatory region are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory region. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of promoter function results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the regulatory region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a regulatory region would be operably joined to a coding sequence if the regulatory region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

Stringent hybridization conditions. Stringent hybridization conditions is a term of art understood by those of ordinary skill in the art. For any given nucleic acid sequence, stringent hybridization conditions are those conditions of temperature, chaotropic acids, buffer, and ionic strength which will permit hybridization of that nucleic acid sequence to its complementary sequence and not to substantially different sequences. The exact conditions which constitute "stringent" conditions, depend upon the nature of the nucleic acid sequence, the length of the sequence, and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which non-specific hybridization occurs to a level at which only specific hybridization is observed, one of ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given sequence to hybridize only with complementary sequences. Suitable ranges of such stringency conditions are described in KRAUSE ET AL., METHODS IN ENZYMOLOGY, 200: 546-56 (1991). Stringent hybridization conditions, depending upon the length and commonality of a sequence, may include temperatures of 20°C-65°C and ionic strengths from 5x to 0.1x SSC. Highly stringent hybridization conditions may include temperatures as low as 40-42°C (when denaturants such as formamide are included) or up to 60-65°C in ionic strengths as low as 0.1x SSC. These ranges, however, are only illustrative and, depending upon the nature of the target sequence, and possible future technological developments, may be more stringent than necessary.

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Less than stringent conditions are employed to isolate nucleic acid sequences which are substantially similar, allelic or homologous to any given sequence.

5     Selectively bind. As used herein with respect to antibodies, an antibody is said to "selectively bind" to a target if the antibody recognizes and binds the target of interest but does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, which includes the target of interest.

10    CalDAG-GEF- or cAMP-GEF-associated disorder, condition, or disease. As used herein, the term "CalDAG-GEF or cAMP-GEF associated disorder, condition, or disease" means any disorder, condition, or disease to which a normal or mutant CalDAG-GEF and/or cAMP-GEF is related in any manner, such as in the causation, prevention, exacerbation, alleviation of the disorder. Thus, as used herein, a CalDAG-GEF- or cAMP-GEF-associated disorder, condition, or disease includes disorders related to the Ras-pathway, such as Ras-related cancers.

15    Adapter protein. As used herein, the term "adapter protein" means any protein that binds or is bound to a CalDAG-GEF or a cAMP-GEF protein, and facilitates localization of the bound CalDAG-GEF or cAMP-GEF at the plasma membrane, thereby facilitating Ras activation.

20    Variant. As used herein a "variant" sequence has, or will result in having, a sufficient amino acid similarity to have a reasonable expectation of success in the methods of the present invention. In order to produce variants of the disclosed sequences that may also functionally serve as a CalDAG-GEF or cAMP-GEF protein, any one or more of the naturally-occurring CalDAG-GEF or cAMP-GEF sequences disclosed herein may be used as a reference sequence to determine whether a candidate sequence possesses sufficient amino acid similarity to have a reasonable expectation of success in the methods of the present invention. Preferably, variant sequences are at least 70% similar or 60% identical, more preferably at least 75% similar or 65% identical, and most preferably 80% similar or 70% identical to one of the disclosed, naturally-occurring sequences.

25    To determine whether a candidate peptide region has the requisite percentage similarity or identity to a reference polypeptide or peptide oligomer, the candidate amino acid sequence and the reference amino acid sequence are first aligned using the dynamic programming algorithm described in Smith and Waterman (1981), J. Mol. Biol. 147:195-197, in combination with the BLOSUM62 substitution matrix described in Figure 2 of Henikoff and Henikoff (1992), "Amino acid substitution matrices from protein blocks", PNAS (1992 Nov), 89:10915-10919. For the present invention, an appropriate value for the gap insertion penalty is -12, and an appropriate

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value for the gap extension penalty is -4. Computer programs performing alignments using the algorithm of Smith-Waterman and the BLOSUM62 matrix, such as the GCG program suite (Oxford Molecular Group, Oxford, England), are commercially available and widely used by those skilled in the art.

5 Once the alignment between the candidate and reference sequence is made, a percent similarity score may be calculated. The individual amino acids of each sequence are compared sequentially according to their similarity to each other. If the value in the BLOSUM62 matrix corresponding to the two aligned amino acids is zero or a negative number, the pairwise similarity score is zero; otherwise the pairwise similarity score is 1.0. The raw similarity score is  
10 the sum of the pairwise similarity scores of the aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent similarity. Alternatively, to calculate a percent identity, the aligned amino acids of each sequence are again compared sequentially. If the amino acids are non-identical, the pairwise identity score is zero; otherwise  
15 the pairwise identity score is 1.0. The raw identity score is the sum of the identical aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent identity. Insertions and deletions are ignored for the purposes of calculating percent similarity and identity. Accordingly, gap penalties are not used in this calculation, although they are used  
20 in the initial alignment.

In all instances, variants of the naturally-occurring CalDAG-GEF or cAMP-GEF proteins, as described above, must be tested for biological activity as described below. Specifically, the proteins must exhibit guanine nucleotide exchange factor activity, and, preferably, they have the ability to inhibit Ras signaling of the Ras/Raf-1/MAP kinase pathway.

25 II. The CalDAG-GEFs

CalDAG-GEFI has a substrate specificity for Rap1A, dual binding domains for calcium ( $\text{Ca}^{2+}$ ) and diacylglycerol (DAG), and enriched expression in brain basal ganglia pathways and their axon-terminal regions. Expression of CalDAG-GEFI activates Rap1A and inhibits Ras-dependent activation of the Erk/MAP kinase cascade in 293T cells.  $\text{Ca}^{2+}$  ionophore and phorbol ester strongly and additively enhance this Rap1A activation. By contrast, CalDAG-GEFII exhibits a different brain expression pattern and fails to activate Rap1A, but activates H-Ras, R-Ras and the Erk/MAP kinase cascade under  $\text{Ca}^{2+}$  and DAG modulation. The CalDAG-GEF

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proteins have a critical neuronal function in determining the relative activation of Ras and Rap1 signaling induced by  $\text{Ca}^{2+}$  and DAG mobilization. The expression of CalDAG-GEFI and CalDAG-GEFII in hematopoietic organs indicates that such control has broad significance in Ras/Rap regulation of normal and malignant states.

5       The basal ganglia are centrally implicated in movement control and in forms of procedural learning related to habit formation. It is not yet known whether particular neurochemical specializations of the basal ganglia contribute to these behavioral functions. The basal ganglia do, however, have a unique double-inhibitory pathway design combined with abundant expression of neuromodulators in striatal neurons. A number of genes with  
10      differentially high expression in the striatum have also been identified. These include genes coding for proteins with signaling functions, such as adenylate cyclase V (Glatt et al., 361 NATURE (LONDON), 536-38 (1993)) and DARPP-32 (Hemmings et al., 310 NATURE (LONDON) 502-05 (1984)). To identify other cellular signaling molecules that could contribute to basal  
15      ganglia functions, a search for striatum-enriched transcripts was performed by a differential display method, as discussed in Example 1. Among the transcripts identified in this search were a family of genes characterized by the presence of a Ras superfamily (GEF) domain.

Specific domains identified include structurally conserved GEF regions SCR1, SCR2, and SCR3, as shown in Figures 2C and 3D, and as shown in the following table.

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TABLE 1

Gene	SCR1	SCR2	SCR3
hCalDAG-GEFI	SEQ ID NO.3: 605-677 SEQ ID NO.4: 149-173	SEQ ID NO.3: 817-946 SEQ ID NO.4: 219-262	SEQ ID NO.3: 1053-1185 SEQ ID NO.4: 298-320
hCalDAG-GEFII	SEQ ID NO.7: 728-800 SEQ ID NO.8: 205-229	SEQ ID NO.7: 913-1042 SEQ ID NO.8: 270-313	SEQ ID NO.7: 1084-1216 SEQ ID NO.8: 348-371
hcAMP-GEFI	SEQ ID NO.11: 2058-2130 SEQ ID NO.12: 205-229	SEQ ID NO.11: 2276-2405 SEQ ID NO.12: 688-731	SEQ ID NO.11: 2516-2582 SEQ ID NO.12: 767-789
rcAMP-GEFI	SEQ ID NO.9: 2050-2122 SEQ ID NO.10: 618-642	SEQ ID NO.9: 2267-2396 SEQ ID NO.10: 691-734	SEQ ID NO.9: 2502-2568 SEQ ID NO.10: 770-792
hcAMP-GEFII	SEQ ID NO.17: 2707-2779 SEQ ID NO.18: 767-791		
rcAMP-GEFII	SEQ ID NO.15: 576-648 SEQ ID NO.16: 192-216		

In addition, the EF hand and DAG-binding domains were identified as shown in Figures 2E and 2F, and as shown in the following table:

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Table 2

Gene	EF Hand Domain	DAG-Binding Domain
hCalDaG-GEFI	SEQ ID NO.3: 1456-1516 SEQ ID NO.4: 432-452	SEQ ID NO.3: 1652-1804 SEQ ID NO.4: 498-548
hCalDAG-GEFII	SEQ ID NO.7: 1384-1444 SEQ ID NO.8: 427-447	SEQ ID NO.7: 1579-1729 SEQ ID NO.8: 492-542

Finally, the cAMP-binding domains were identified as shown in Figure 3E, and as shown in the following table:

5

Table 3

Gene	cAMP-Binding Domain
hcAMP-GEFI	SEQ ID NO.11: 2012-2255 SEQ ID NO.12: 219-300
rcAMP-GEFI	SEQ ID NO.9: 853-1096 SEQ ID NO.10: 219-300
rcAMP-GEFII	SEQ ID NO.17: 1522-1765 SEQ ID NO.18: 372-453

### III. The cAMP-GEFs

Cyclic adenosine 3', 5'-monophosphate (cAMP) is a universal second messenger that induces a variety of physiological responses in eukaryotic cells ranging from growth, differentiation, and gene expression to secretion and neurotransmission. The cAMP second messenger system has also been centrally implicated in modulating synaptic function, neuroplasticity and learning and memory. Most of these effects have been attributed to the binding of cAMP to cAMP-dependent protein kinase (PKA), leading in turn to the activation of

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intracellular phosphorylation cascades. Reported herein is the identification of a new family of cAMP binding proteins that are differentially distributed in the brain and body organs and that are characterized by the presence of both a cAMP binding domain and a guanine nucleotide exchange factor (GEF) domain. These proteins, cAMP-GEFs, bind cAMP and selectively 5 activate the Ras superfamily small G protein, Rap1A, in a cAMP-dependent but PKA-independent manner.

The general concept of cAMP signaling involves the sequential activation (or inhibition) of cAMP production by G proteins, the binding of cAMP to PKA, and the triggering of a series of downstream serine-threonine phosphorylation cascades. Viewed as the nearly exclusive 10 target of cAMP binding in eukaryotic cells, PKA has been considered the essential effector molecule mediating a wide range of physiological effects of G protein/cAMP-triggered phosphorylation cascades. As the main cAMP effector, PKA has also been shown to function in the indirect coupling of the cAMP signal transduction system to other intracellular signaling cascades. The cAMP signaling system has also been strongly implicated in neuronal functions 15 ranging from neurotransmitter-initiated signaling to neuroplasticity underlying development and memory, but PKA has not been clearly linked to all of these neuronal functions, and region-specific neuronal effects have been observed as well. The cAMP-GEF gene has a Ras superfamily GEF motif. Thus, the gene codes for a novel cAMP binding protein that directly couples the cAMP signal transduction system to Ras superfamily cascades.

20 IV. Preferred Embodiments

Based, in part, upon the discoveries disclosed and described herein, the following preferred embodiments of the present invention are provided.

1. Isolated Nucleic Acids

In one series of embodiments, the present invention provides isolated nucleic acids 25 corresponding to, or relating to, the CalDAG-GEF or cAMP-GEF nucleic acid sequences disclosed herein. As described more fully below, these sequences include normal CalDAG-GEF and cAMP-GEF sequences from humans and other mammalian species, mutant CalDAG-GEF and cAMP-GEF sequences from humans and other mammalian species, homologous sequences from non-mammalian species such as *Drosophila* and *C. elegans*, subsets of these sequences 30 useful as probes and PCR primers, subsets of these sequences encoding fragments of the CalDAG-GEF or cAMP-GEF proteins or corresponding to particular domains or polymorphic

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- regions, complementary or antisense sequences corresponding to fragments of the CalDAG-GEF or cAMP-GEF genes, sequences in which the CalDAG-GEF and/or cAMP-GEF coding regions have been operably joined to exogenous regulatory regions, and sequences encoding fusion proteins of the portions of the CalDAG-GEF or cAMP-GEF proteins fused to other proteins  
5 useful as markers of expression, as "tags" for purification, or in screens and assays for proteins interacting with the CalDAG-GEFs and/or cAMP-GEFs.

Thus, in a first series of embodiments, isolated nucleic acid sequences are provided which encode normal versions of the CalDAG-GEF and cAMP-GEF proteins. Examples of such nucleic acid sequences are disclosed herein. These nucleic acids may be genomic sequences or 10 may be cDNA sequences (e.g., SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, and 17). Thus, for example, the invention provides nucleic acid sequences in which the alternative splice variants described herein are incorporated at the DNA level, thereby, enabling cells including these sequences to express only one of the alternative splice variants at each splice position. For 15 example, a recombinant gene may be produced in which one of the splice variants of cAMP-GEF is incorporated into DNA such that cells having this recombinant gene can express only one of these variants. For purposes of reducing the size of a recombinant CalDAG-GEF or cAMP-GEF gene, a cDNA gene may be employed or various combinations of the introns and untranslated exons may be removed from a DNA construct. Such constructs may be particularly useful, as described below, in identifying compounds which can induce or repress the expression of the 20 CalDAG-GEF or cAMP-GEF genes.

In addition to the disclosed CalDAG-GEF and/or cAMP-GEF sequences, one of ordinary skill in the art is now enabled to identify and isolate nucleic acids corresponding to CalDAG-GEF or cAMP-GEF genes or cDNAs which are allelic to the disclosed sequences or which are heterospecific homologues. Thus, the present invention provides isolated nucleic acids 25 corresponding to these alleles and homologues, as well as various recombinant constructs derived from these sequences, by means which are well known in the art. Briefly, one of ordinary skill in the art may now screen preparations of genomic or cDNA, including samples prepared from individual organisms (e.g., human cancer patients or their family members) as well as bacterial, viral, yeast or other libraries of genomic or cDNA, using probes or PCR 30 primers to identify allelic or homologous sequences. Because it is desirable to identify additional CalDAG-GEF and/or cAMP-GEF gene mutations which may contribute to the development of Ras-related cancers, because it is desirable to identify additional CalDAG-GEF and/or cAMP-

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GEF polymorphisms which are not mutant or have antitumorigenic effects, and because it is also desired to create a variety of animal models which may be used to study Ras-related cancers and screen for potential therapeutics, it is particularly contemplated that additional CalDAG-GEF and/or cAMP-GEF sequences will be isolated from other preparations or libraries of human nucleic acids and from preparations or libraries from animals including rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates. Furthermore, 5 CalDAG-GEF and/or cAMP-GEF homologues from yeast or invertebrate species, including *C. elegans* and other nematodes, as well as *Drosophila* and other insects, may have particular utility for drug screening. For example, invertebrates bearing mutant CalDAG-GEF and/or cAMP-GEF 10 homologues (or mammalian CalDAG-GEF and/or cAMP-GEF transgenes) which cause a rapidly occurring and easily scored phenotype (e.g., abnormal eye development after several days) can be used as screens for drugs which block the effect of the mutant gene. Such invertebrates may prove far more rapid and efficient for mass screenings than larger vertebrate animals. Once lead compounds are found through such screens, they may be tested in higher animals.

15 Depending upon the intended use, the present invention provides nucleic acid subsequences of the CalDAG-GEF and/or cAMP-GEF genes which may have lengths varying from 8-10 nucleotides (e.g., for use as PCR primers) to nearly the full size of the CalDAG-GEF and/or cAMP-GEF genes. Thus, the present invention provides isolated nucleic acids comprising sequences corresponding to at least 8, preferably at least 10, and more preferably at least 15 consecutive nucleotides of the CalDAG-GEF and/or cAMP-GEF genes, as disclosed or otherwise 20 enabled herein, or to their complements.

In another series of embodiments, the present invention provides for isolated nucleic acids encoding all or a portion of the CalDAG-GEF and/or cAMP-GEF proteins in the form of a fusion protein. In these embodiments, a nucleic acid regulatory region (endogenous or 25 exogenous) is operably joined to a first coding region which is covalently joined in-frame to a second coding region. The CalDAG-GEF and/or cAMP-GEF sequences of the fusion protein may represent the first, second, or any additional coding regions. The CalDAG-GEF and/or cAMP-GEF sequences may be conserved or non-conserved domains and can be placed in any coding region for the fusion protein.

30 In another series of embodiments, the present invention provides isolated nucleic acids in the form of recombinant DNA constructs in which a marker or reporter gene (e.g.,  $\beta$ -galactosidase, luciferase) is operably joined to the 5' regulatory region of a CalDAG-GEF and/or

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cAMP-GEF gene such that expression of the marker gene is under the control of the CalDAG-GEF and/or cAMP-GEF regulatory sequences. Such isolated nucleic acids may be used to produce cells, cell lines or transgenic animals which are useful in the identification of compounds which can, directly or indirectly, differentially affect the expression of the CalDAG-  
5 GEFs and/or cAMP-GEFs.

Finally, the isolated nucleic acids of the present invention include any of the above described sequences when included in vectors. Appropriate vectors include cloning vectors and expression vectors of all types, including plasmids, phagemids, cosmids, episomes, and the like, as well as integration vectors. The vectors may also include various marker genes (e.g.,  
10 antibiotic resistance or susceptibility genes) which are useful in identifying cells successfully transformed therewith. In addition, the vectors may include regulatory sequences to which the nucleic acids of the invention are operably joined, and/or may also include coding regions such that the nucleic acids of the invention, when appropriately ligated into the vector, are expressed as fusion proteins. Such vectors may also include vectors for use in yeast "two hybrid,"  
15 baculovirus, and phage-display systems.

## 2. Substantially Pure Proteins

The present invention provides for substantially pure preparations of the CalDAG-GEF and/or cAMP-GEF proteins, fragments of the CalDAG-GEF and/or cAMP-GEF proteins, and fusion proteins including the CalDAG-GEFs and/or cAMP-GEFs or fragments thereof. The  
20 proteins, fragments and fusions have utility, as described herein, in the generation of antibodies to normal and mutant CalDAG-GEFs and/or cAMP-GEFs, in the identification of CalDAG-GEF and/or cAMP-GEF binding proteins, and in diagnostic and therapeutic methods. Therefore,  
depending upon the intended use, the present invention provides substantially pure proteins or peptides comprising amino acid sequences which are subsequences of the complete CalDAG-  
25 GEF and/or cAMP-GEF proteins and which may have lengths varying from 4-8 amino acids (e.g., for use as immunogens), or 9-15 amino acids (e.g., for use in binding assays), to the complete CalDAG-GEF and/or cAMP-GEF proteins. Thus, the present invention provides substantially pure proteins or peptides comprising sequences corresponding to at least 4, preferably at least 9, more preferably at least 15 consecutive amino acids of the CalDAG-GEF  
30 and/or cAMP-GEF proteins, as disclosed or otherwise enabled herein.

Purification can be achieved using standard protein purification procedures including, but not limited to, gel-filtration chromatography, ion-exchange chromatography, high-performance

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liquid chromatography (RP-HPLC, ion-exchange HPLC, size-exclusion HPLC, high-performance chromatofocusing chromatography, hydrophobic interaction chromatography, immunoprecipitation, or immunoaffinity purification. Gel electrophoresis (e.g., PAGE, SDS-PAGE) can also be used to isolate a protein or peptide based on its molecular weight, charge properties, and hydrophobicity.

A CalDAG-GEF or cAMP-GEF protein, or a fragment thereof, may also be conveniently purified by creating a fusion protein including the desired CalDAG-GEF or cAMP-GEF sequence fused to another peptide such as an antigenic determinant or poly-His tag (e.g., QIAexpress vectors, (QIAGEN Corp., Chatsworth, CA)), or a larger protein (e.g., GST using the pGEX-27 vector (Amrad, USA) or green fluorescent protein using the Green Lantern vector (GIBCO/BRL, Gaithersburg, MD)).

### 3. Antibodies to the CalDAG-GEF and/or cAMP-GEFs

The present invention also provides antibodies, and methods of making antibodies, which selectively bind to the CalDAG-GEF and/or cAMP-GEF proteins or fragments thereof. The antibodies of the invention have utility as laboratory reagents for, *inter alia*, immunoaffinity purification of the CalDAG-GEFs and/or cAMP-GEFs, Western blotting to identify cells or tissues expressing the CalDAG-GEFs and/or cAMP-GEFs, and immunocytochemistry or immunofluorescence techniques to establish the subcellular location of the protein.

The antibodies of the invention may be generated in a host using the entire CalDAG-GEF and/or cAMP-GEF proteins of the invention or using any CalDAG-GEF and/or cAMP-GEF epitope which is characteristic of that protein and which substantially distinguishes it from host proteins. Such epitopes may be identified by comparing sequences of, for example, 4-8 amino acid residues from a CalDAG-GEF and/or cAMP-GEF sequence to computer databases of protein sequences from the relevant host. Antibodies against highly conserved domains are expected to have the greatest utility for purification or identification of CalDAG-GEFs and/or cAMP-GEFs.

Amino acid residue positions which are potential antigenic sites in the CalDAG-GEF or cAMP-GEF proteins and which may be useful in generating the antibodies of the invention may be determined by using computer programs such as the IBI Pustell program. Other methods of choosing antigenic determinants are known in the art and may, of course, be employed. In addition, larger fragments (e.g., 9-15 residues) including some of these epitopes may also be employed. Even larger fragments, including, for example, entire functional domains or multiple

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functional domains may also be preferred. For an overview of antibody techniques, see Antibody Engineering: A Practical Guide, Borrebaek, ed., W.H. Freeman & Company, NY (1992), or Antibody Engineering, 2nd Ed., Borrebaek, ed., Oxford University Press, Oxford (1995).

- 5 The antibodies of the invention may be labelled or conjugated with other compounds or materials for diagnostic and/or therapeutic uses. For example, they may be coupled to radionuclides, fluorescent compounds, or enzymes for imaging or therapy, or to liposomes for the targeting of compounds contained in the liposomes to a specific tissue location.

#### 4. Transformed Cell Lines

- The present invention also provides for cells or cell lines, both prokaryotic and eukaryotic, which have been transformed or transfected with the nucleic acids of the present invention so as to cause clonal propagation of those nucleic acids and/or expression of the proteins or peptides encoded thereby. Such cells or cell lines will have utility not only in the propagation and production of the nucleic acids and proteins of the present invention but also, as further described herein, as model systems for diagnostic and therapeutic assays. As used herein, 10 the term "transformed cell" is intended to embrace any cell, or the descendant of any cell, into which has been introduced any of the nucleic acids of the invention, whether by transformation, transfection, infection, electroporation, microinjection or other means. Methods of producing appropriate vectors, transforming cells with those vectors, and identifying transformants are well known in the art and are only briefly reviewed here (see, for example, Sambrook et al. (1989) 15 Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

- Vectors may be introduced into the recipient or "host" cells by various methods well known in the art including, but not limited to, calcium phosphate transfection, strontium phosphate transfection, DEAE dextran transfection, electroporation, lipofection (e.g., Dospel 20 Liposomal transfection reagent, Boehringer Mannheim, Germany), microinjection, ballistic insertion on micro-beads, protoplast fusion or, for viral or phage vectors, by infection with the recombinant virus or phage.

#### 5. Transgenic Animal Models

- The present invention also provides for the production of transgenic non-human animal models for the study of Ras-related cancers, for the screening of candidate pharmaceutical 30 compounds, for the creation of explanted mammalian cell cultures (e.g., neuronal, glial,

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organotypic or mixed cell cultures) in which mutant or wild type CalDAG-GEF and/or cAMP-GEF sequences are expressed or in which the CalDAG-GEF and/or cAMP-GEF genes have been inactivated (*e.g.*, "knock-out" deletions), and for the evaluation of potential therapeutic interventions.

5 Species suitable for use as animal models in the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates (*e.g.*, Rhesus monkeys, chimpanzees).

10 Various techniques for generating transgenic animals, as well as techniques for homologous recombination or gene targeting, are now widely accepted and practiced. See, for example, Hogan et al., *Manipulating Mouse Embryo* (1986). To create a transgene, the target sequence of interest (*e.g.*, mutant or wild-type CalDAG-GEF or cAMP-GEF sequences) is typically ligated into a cloning site located downstream of a promoter element which will regulate the expression of RNA from the CalDAG-GEF or cAMP-GEF sequence. An alternate approach to creating a transgene is to use endogenous CalDAG-GEF or cAMP-GEF regulatory sequences to drive expression of the CalDAG-GEF or cAMP-GEF transgene.

15 6. Assays for Drugs Which Affect CalDAG-GEF and/or cAMP-GEF Expression

In another series of embodiments, the present invention provides assays for identifying small molecules or other compounds which are capable of inducing or inhibiting the expression of the CalDAG-GEF or cAMP-GEF genes and proteins. The assays may be performed *in vitro* using non-transformed cells, immortalized cell lines, or recombinant cell lines, or *in vivo* using the transgenic animal models enabled herein.

20 In particular, the assays may detect the presence of increased or decreased expression of CalDAG-GEF, cAMP-GEF, or other CalDAG-GEF or cAMP-GEF-related genes or proteins, on the basis of increased or decreased mRNA expression (using, *e.g.*, the nucleic acid probes disclosed and enabled herein), increased or decreased levels of CalDAG-GEF, cAMP-GEF or other CalDAG-GEF or cAMP-GEF-related protein products (using, *e.g.*, the anti-CalDAG-GEF or anti-cAMP-GEF antibodies disclosed and enabled herein), or increased or decreased levels of expression of a marker gene (*e.g.*,  $\beta$ -galactosidase or luciferase) operably joined to a CalDAG-GEF or cAMP-GEF 5' regulatory region in a recombinant construct.

25 Thus, for example, one may culture cells known to express a particular CalDAG-GEF or cAMP-GEF and add to the culture medium one or more test compounds. After allowing a sufficient period of time (*e.g.*, 0-72 hours) for the compound to induce or inhibit the expression

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of the CalDAG-GEF or cAMP-GEF, any change in levels of expression from an established baseline may be detected using any of the techniques described above and well known in the art. In particularly preferred embodiments, the cells are from an immortalized cell line such as a human neuroblastoma, glioblastoma or a hybridoma cell line. Using the nucleic acid probes and/or antibodies disclosed and enabled herein, detection of changes in the expression of a CalDAG-GEF or cAMP-GEF and thus, identification of the compound as an inducer or repressor of CalDAG-GEF and/or cAMP-GEF expression, requires only routine experimentation.

In particularly preferred embodiments, a recombinant assay is employed in which a reporter gene such as a  $\beta$ -galactosidase, green fluorescent protein, alkaline phosphatase, or luciferase is operably joined to a 5' regulatory region of a CalDAG-GEF or cAMP-GEF gene. The reporter gene and regulatory regions are joined in-frame (or in each of the three possible reading frames) so that transcription and translation of the reporter gene may proceed under the control of the CalDAG-GEF or cAMP-GEF regulatory elements. The recombinant construct may then be introduced into any appropriate cell type, although mammalian cells are preferred, and human cells are most preferred. The transformed cells may be grown in culture and, after establishing the baseline level of expression of the reporter gene, test compounds may be added to the medium. The ease of detection of the expression of the reporter gene provides for a rapid, high through-put assay for the identification of inducers and repressors of the CalDAG-GEF or cAMP-GEF gene.

Compounds identified by this method will have potential utility in modifying the expression of the CalDAG-GEF, cAMP-GEF or other CalDAG-GEF or cAMP-GEF-related genes *in vivo*. These compounds may be further tested in the animal models disclosed and enabled herein to identify those compounds having the most potent *in vivo* effects. In addition, as described herein with respect to small molecules having CalDAG-GEF or cAMP-GEF-binding activity, these molecules may serve as "lead compounds" for the further development of pharmaceuticals by, for example, subjecting the compounds to sequential modifications, molecular modeling, and other routine procedures employed in rational drug design.

#### 7. Identification of Compounds with CalDAG-GEF and/or cAMP-GEF Binding Capacity

In light of the present disclosure, one of ordinary skill in the art is enabled to practice new screening methodologies which will be useful in the identification of proteins and other compounds which bind to, or otherwise directly interact with, the CalDAG-GEFs or cAMP-GEFs. The proteins and compounds will include endogenous cellular components which interact

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with the CalDAG-GEFs or cAMP-GEFs *in vivo* and which, therefore, provide new targets for pharmaceutical and therapeutic interventions, as well as recombinant, synthetic and otherwise exogenous compounds which may have CalDAG-GEF or cAMP-GEF binding capacity and, therefore, may be candidates for pharmaceutical agents. Thus, in one series of embodiments, cell lysates or tissue homogenates (e.g., human brain homogenates, leukocyte lysates) may be screened for proteins or other compounds which bind to one of the normal or mutant CalDAG-GEFs and/or cAMP-GEFs. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (e.g., libraries of small molecules or peptides), may be screened for CalDAG-GEF or cAMP-GEF binding capacity. Small molecules are particularly preferred in this context because they are more readily absorbed after oral administration, have fewer potential antigenic determinants, and/or are more likely to cross the blood brain barrier than larger molecules such as nucleic acids or proteins. The methods of the present invention are particularly useful in that they may be used to identify molecules which selectively or preferentially bind to a mutant form of a CalDAG-GEF or cAMP-GEF protein (rather than a normal form) and, therefore, may have particular utility in treating the heterozygous victims of a CalDAG-GEF or cAMP-GEF associated disorder.

Compounds which bind to normal, mutant or both forms of the CalDAG-GEFs or cAMP-GEFs may have utility in treatments and diagnostics. Compounds which bind only to a normal CalDAG-GEF or cAMP-GEF may, for example, act as enhancers of its normal activity and thereby at least partially compensate for the lost or abnormal activity of mutant forms of the CalDAG-GEF or cAMP-GEF in victims suffering from CalDAG-GEF- or cAMP-GEF-associated disorders. Compounds which bind to both normal and mutant forms of a CalDAG-GEF or cAMP-GEF may have utility if they differentially affect the activities of the two forms so as to alleviate the overall departure from normal function. Alternatively, blocking the activity of both normal and mutant forms of either CalDAG-GEF or cAMP-GEF may have less severe physiological and clinical consequences than the normal progress of the disorder and, therefore, compounds which bind to and inhibit the activity of both normal and mutant forms of a CalDAG-GEF or cAMP-GEF may be therapeutically useful. Preferably, however, compounds are identified which have a higher affinity of binding to mutant CalDAG-GEF or cAMP-GEF than to normal CalDAG-GEF or cAMP-GEF, and which selectively or preferentially inhibit the activity of the mutant form. Such compounds may be identified by using any of the techniques

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described herein, and then comparing the binding affinities of the candidate compound(s) for the normal and mutant forms of CalDAG-GEF or cAMP-GEF.

The effect of agents which bind to the CalDAG-GEFs or cAMP-GEFs (normal or mutant forms of either) can be monitored either by direct monitoring of this binding (e.g., using the

- 5 BIACore assay, LKB Pharmacia, Sweden) or by indirect monitoring of binding by detecting, for example, a change in fluorescence, molecular weight, or concentration of either the binding agent or CalDAG-GEF or cAMP-GEF component, either in a soluble phase or in a substrate-bound phase.

Once identified by the methods described above, the candidate compounds may then be  
10 produced in quantities sufficient for pharmaceutical administration or testing (e.g.,  $\mu$ g or mg or greater quantities), and formulated in a pharmaceutically acceptable carrier (see, e.g., REMINGTON'S PHARMACEUTICAL SCIENCES, Gennaro, A., ed., Mack Pub., (1990)). These candidate compounds may then be administered to the transformed cells of the invention, to the transgenic animal models of the invention, to cell lines derived from the animal models or from  
15 human patients, or to patients with CalDAG-GEF- or cAMP-GEF-associated disorders. The animal models described and enabled herein are of particular utility in further testing candidate compounds which bind to normal or mutant CalDAG-GEF or cAMP-GEF for their therapeutic efficacy.

In addition, once identified by the methods described above, the candidate compounds  
20 may also serve as "lead compounds" in the design and development of new pharmaceuticals. For example, as is well known in the art, sequential modification of small molecules (e.g., amino acid residue replacement for peptides; functional group replacement for peptide or non-peptide compounds) is a standard approach in the pharmaceutical industry for the development of new pharmaceuticals. Such development generally proceeds from a "lead compound" which is shown  
25 to have at least some of the activity (e.g., CalDAG-GEF or cAMP-GEF binding or blocking ability) of the desired pharmaceutical. In particular, when one or more compounds having at least some activity of interest (e.g., modulation of CalDAG-GEF or cAMP-GEF activity) are identified, structural comparison of the molecules can greatly inform the skilled practitioner by suggesting portions of the lead compounds which should be conserved and portions which may  
30 be varied in the design of new candidate compounds. Thus, the present invention also provides a means of identifying lead compounds which may be sequentially modified to produce new candidate compounds for use in the treatment of CalDAG-GEF- or cAMP-GEF-associated

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disorders. These new compounds then may be tested both for CalDAG-GEF or cAMP-GEF-binding or blocking (e.g., in the binding assays described above) and for therapeutic efficacy (e.g., in the animal models described herein). This procedure may be iterated until compounds having the desired therapeutic activity and/or efficacy are identified.

5 In each of the present series of embodiments, an assay is conducted to detect binding between a "CalDAG-GEF component" or a "cAMP-GEF component" and some other moiety. Of particular utility will be sequential assays in which compounds are tested for the ability to bind to only the normal or only the mutant forms of the CalDAG-GEF or cAMP-GEF functional domains using mutant and normal CalDAG-GEF or cAMP-GEF components in the binding  
10 assays. Such compounds are expected to have the greatest therapeutic utilities, as described more fully below. The "CalDAG-GEF component" or the "cAMP-GEF component" in these assays may be a complete normal or mutant form of a CalDAG-GEF or cAMP-GEF protein (e.g., an hCalDAG-GEF or hcAMP-GEF variant) but need not be. Rather, particular functional domains of the CalDAG-GEFs or cAMP-GEFs, as described above, may be employed either as  
15 separate molecules or as part of a fusion protein. For example, to isolate proteins or compounds that interact with these functional domains, screening may be carried out using fusion constructs and/or synthetic peptides corresponding to these regions. Obviously, various combinations of fusion proteins and functional domains from CalDAG-GEF or cAMP-GEF are possible. In addition, the functional domains may be altered so as to aid in the assay by, for example,  
20 introducing into the functional domain a reactive group or amino acid residue (e.g., cysteine) which will facilitate immobilization of the domain on a substrate (e.g., using sulphydryl reactions).

Methods for screening cellular lysates, tissue homogenates, or small molecule libraries for candidate CalDAG-GEF or cAMP-GEF-binding molecules are well known in the art and, in  
25 light of the present disclosure, may now be employed to identify compounds which bind to normal or mutant CalDAG-GEF or cAMP-GEF components or which modulate CalDAG-GEF or cAMP-GEF activity as defined by non-specific measures (e.g., changes in intracellular Ca<sup>2+</sup>, GTP/GDP ratio) or by specific measures (e.g., changes in the expression of other downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential  
30 hybridization, or SAGE methods). The preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-isolation of CalDAG-GEF or cAMP-GEF components and bound proteins or other compounds by immunoprecipitation; (3)

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the Biomolecular Interaction Assay (BIAcore); and (4) the yeast two-hybrid systems. These and others are discussed separately below.

A. Affinity Chromatography

In light of the present disclosure, a variety of affinity binding techniques well known in the art may be employed to isolate proteins or other compounds which bind to the CalDAG-GEFs or cAMP-GEFs disclosed or otherwise enabled herein. In general, a CalDAG-GEF or cAMP-GEF component may be immobilized on a substrate (*e.g.*, a column or filter) and a solution including the test compound(s) is contacted with the CalDAG-GEF or cAMP-GEF protein, fusion or fragment under conditions which are permissive for binding. The substrate is then washed with a solution to remove unbound or weakly bound molecules. A second wash may then elute those compounds which strongly bound to the immobilized normal or mutant CalDAG-GEF or cAMP-GEF component. Alternatively, the test compounds may be immobilized and a solution containing one or more CalDAG-GEF or cAMP-GEF components may be contacted with the column, filter, or other substrate. The ability of the CalDAG-GEF or cAMP-GEF component to bind to the test compounds may be determined as above or a labeled form of the CalDAG-GEF or cAMP-GEF component (*e.g.*, a radio-labeled or chemiluminescent functional domain) may be used to more rapidly assess binding to the substrate-immobilized compound(s).

B. Co-Immunoprecipitation

Another well characterized technique for the isolation of the CalDAG-GEF or cAMP-GEF components and their associated proteins or other compounds is direct immunoprecipitation with antibodies. This procedure has been successfully used, for example, to isolate many of the synaptic vesicle associated proteins (Phizicky et al., 59 J. BIOL. CHEM. 94-123 (1994)). Thus, either normal or mutant CalDAG-GEF or cAMP-GEF components may be mixed in a solution with the candidate compound(s) under conditions which are permissive for binding, and the CalDAG-GEF or cAMP-GEF component may be immunoprecipitated. Proteins or other compounds which co-immunoprecipitate with the CalDAG-GEF or cAMP-GEF component may then be identified by standard techniques as described above. General techniques for immunoprecipitation may be found in, for example, Harlow et al., ANTIBODIES: A LABORATORY MANUAL (1988).

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The antibodies employed in this assay, as described and enabled herein, may be polyclonal or monoclonal, and include the various antibody fragments as well as single chain antibodies, and the like.

5       C. The Biomolecular Interaction Assay

Another useful method for the detection and isolation of binding proteins is the Biomolecular Interaction Assay or "BIAcore" system developed by Pharmacia Biosensor and described in the manufacturer's protocol (LKB Pharmacia, Sweden). In light of the present disclosure, one of ordinary skill in the art is now enabled to employ this system, or a substantial equivalent, to identify proteins or other compounds having CalDAG-GEF or cAMP-GEF binding capacity. The BIAcore system uses an affinity purified anti-GST antibody to immobilize GST-fusion proteins onto a sensor chip. Obviously, other fusion proteins and corresponding antibodies may be substituted. The sensor utilizes surface plasmon resonance which is an optical phenomenon that detects changes in refractive indices. A homogenate of a tissue of interest is passed over the immobilized fusion protein and protein-protein interactions are registered as changes in the refractive index. This system can be used to determine the kinetics of binding and to assess whether any observed binding is of physiological relevance.

10      D. The Yeast Two-Hybrid System

15      The yeast "two-hybrid" system takes advantage of transcriptional factors that are composed of two physically separable, functional domains. One commonly used system employs the yeast GAL4 transcriptional activator, consisting of a DNA binding domain and a transcriptional activation domain. Two different cloning vectors are used to generate separate fusions of the GAL4 domains to genes encoding potential binding proteins. The fusion proteins are co-expressed, targeted to the nucleus and, if interactions occur, activation of a reporter gene (e.g., lacZ) produces a detectable phenotype.

20      E. Other Methods

25      The nucleotide sequences and protein products, including both mutant and normal forms of these nucleic acids and their corresponding proteins, can be used with the above techniques to isolate other interacting proteins, and to identify other genes whose expression is altered by the over-expression of normal CalDAG-GEF or cAMP-GEF sequences, by the under-expression of normal CalDAG-GEFs or cAMP-GEFs sequences, or by the expression of mutant CalDAG-GEF and/or cAMP-GEF sequences. Identification of these interacting proteins, as well as the identification of other genes whose expression levels are altered in the face of mutant CalDAG-

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- GEF or cAMP-GEF sequences (for instance) will identify other gene targets which have direct relevance to the pathogenesis of this disease in its clinical or pathological forms. Specifically, these techniques rely on PCR-based and/or hybridization-based methods to identify genes which are differentially expressed between two conditions (a cell line expressing normal CalDAG-  
5 GEFs or cAMP-GEFs compared to the same cell type expressing a mutant CalDAG-GEF or cAMP-GEF sequence). These techniques include differential display, serial analysis of gene expression (SAGE), mass-spectrometry of protein, 2D-gels and subtractive hybridization (*See, e.g.*, Nowak, 270 Sci. 368-371 (1995); Kahn, 270 Sci. 369-370 (1995)).
8. Methods of Identifying Compounds Modulating CalDAG-GEF and/or cAMP-GEF Activity  
10 In another series of embodiments, the present invention provides for methods of identifying compounds with the ability to modulate the activity of normal and mutant CalDAG-GEFs and/or cAMP-GEFs. As used with respect to this series of embodiments, the term “activity” broadly includes gene and protein expression, CalDAG-GEF and/or cAMP-GEF protein post-translation processing, trafficking and localization, and any functional activity (*e.g.*, enzymatic, receptor-effector, binding, channel), as well as downstream affects of any of these.  
15 Using the transformed cells and transgenic animal models of the present invention, cells obtained from subjects bearing a mutant CalDAG-GEF and/or cAMP-GEF gene, or animals or human subjects bearing naturally occurring CalDAG-GEF and/or cAMP-GEF mutations, it is now possible to screen candidate pharmaceuticals and treatments for their therapeutic effects by  
20 detecting changes in one or more of the functional characteristics or phenotypic manifestations of normal or mutant CalDAG-GEF and/or cAMP-GEF expression.

Thus, the present invention provides methods for screening or assaying for proteins, small molecules or other compounds which modulate CalDAG-GEF and/or cAMP-GEF activity by contacting a cell *in vivo* or *in vitro* with a candidate compound and assaying for a change in a  
25 marker associated with normal or mutant CalDAG-GEF and/or cAMP-GEF activity. The marker associated with CalDAG-GEF and/or cAMP-GEF activity may be any measurable biochemical, physiological, histological and/or behavioral characteristic associated with CalDAG-GEF and/or cAMP-GEF expression. In particular, useful markers will include any measurable biochemical, physiological, histological and/or behavioral characteristic which distinguishes cells, tissues,  
30 animals or individuals bearing at least one mutant CalDAG-GEF and/or cAMP-GEF gene from their normal counterparts. In addition, the marker may be any specific or non-specific measure of CalDAG-GEF and/or cAMP-GEF activity, such as the GDP/GTP bound to Rap1/Ras.

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CalDAG-GEF and/or cAMP-GEF specific measures include measures of CalDAG-GEF and/or cAMP-GEF expression (e.g., CalDAG-GEF and/or cAMP-GEF mRNA or protein levels) which may employ the nucleic acid probes or antibodies of the present invention. Non-specific measures include changes in cell physiology such as pH, intracellular calcium, cAMP levels, 5 overall GTP/GDP ratios, phosphatidylinositol activity, protein phosphorylation, etc., which can be monitored by known methods. The activation or inhibition of CalDAG-GEF or cAMP-GEF activity in its mutant or normal form can also be monitored by examining changes in the expression of other genes which are specific to the CalDAG-GEF and/or cAMP-GEF pathway. These can be assayed by such techniques as differential display, differential hybridization, and 10 SAGE, as well as by 2-D gel electrophoresis of cellular lysates. In each case, the differentially-expressed genes can be ascertained by inspection of identical studies before and after application of the candidate compound. Furthermore, as noted elsewhere, the particular genes whose expression is modulated by the administration of the candidate compound can be ascertained by cloning, nucleotide sequencing, amino acid sequencing, or mass spectrometry.

15 In general, a cell may be contacted with a candidate compound and, after an appropriate period (e.g., 0-72 hours for most biochemical measures of cultured cells), the marker of CalDAG-GEF or cAMP-GEF activity may be assayed and compared to a baseline measurement. The baseline measurement may be made prior to contacting the cell with the candidate compound or may be an external baseline established by other experiments or known in the art. 20 The cell may be a transformed cell of the present invention or an explant from an animal or individual. In particular, the cell may be an explant from a carrier of a CalDAG-GEF or cAMP-GEF mutation or an animal model of the invention (e.g., a transgenic nematode or mouse bearing a mutant CalDAG-GEF or cAMP-GEF gene). Preferred cells include those from neurological tissues such as neuronal, glial or mixed cell cultures; and cultured fibroblasts, liver, kidney, 25 spleen, or bone marrow. The cells may be contacted with the candidate compounds in a culture *in vitro* or may be administered *in vivo* to a live animal or human subject. For live animals or human subjects, the test compound may be administered orally or by any parenteral route suitable to the compound. For clinical trials of human subjects, measurements may be conducted periodically (e.g., daily, weekly or monthly) for several months or years.

30 In light of the identification, characterization, and disclosure herein of the CalDAG-GEF or cAMP-GEF genes and proteins, the CalDAG-GEF or cAMP-GEF nucleic acid probes and antibodies, and the CalDAG-GEF or cAMP-GEF transformed cells and transgenic animals of the

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invention, one of ordinary skill in the art is now enabled by perform a great variety of assays which will detect the modulation of CalDAG-GEF or cAMP-GEF activity by candidate compounds. Particularly preferred and contemplated embodiments are discussed in some detail below.

5        A. CalDAG-GEF and/or cAMP-GEF Expression

In one series of embodiments, specific measures of CalDAG-GEF or cAMP-GEF expression are employed to screen candidate compounds for their ability to affect CalDAG-GEF or cAMP-GEF activity. Thus, using the CalDAG-GEF or cAMP-GEF nucleic acids and antibodies disclosed and otherwise enabled herein, one may use mRNA levels or protein levels 10 as a marker for the ability of a candidate compound to modulate CalDAG-GEF or cAMP-GEF activity. The use of such probes and antibodies to measure gene and protein expression is well known in the art and discussed elsewhere herein.

B. Intracellular Localization

In another series of embodiments, compounds may be screened for their ability to 15 modulate the activity of the CalDAG-GEFs or cAMP-GEFs based upon their effects on the trafficking and intracellular localization of the CalDAG-GEFs or cAMP-GEFs. Differences in localization of mutant and normal CalDAG-GEFs and/or cAMP-GEFs may contribute to the etiology of CalDAG-GEF and/or cAMP-GEF-associated diseases. Compounds which can affect the localization of the CalDAG-GEFs and/or cAMP-GEFs may, therefore, be identified as 20 potential therapeutics. Standard techniques known in the art may be employed to detect the localization of the CalDAG-GEFs and/or cAMP-GEFs. Generally, these techniques will employ the antibodies of the present invention, and in particular antibodies which selectively bind to one or more mutant CalDAG-GEFs or cAMP-GEFs but not to normal CalDAG-GEFs or cAMP-GEFs. As is well known in the art, such antibodies may be labeled by any of a variety of 25 techniques (e.g., fluorescent or radioactive tags, labeled secondary antibodies, avidin-biotin, etc.) to aid in visualizing the intracellular location of the CalDAG-GEFs or cAMP-GEFs. The CalDAG-GEFs or cAMP-GEFs may be co-localized to particular structures, as is known in the art, using antibodies to markers of those structures (e.g., TGN38 for the Golgi, transferrin receptor for post-Golgi transport vesicles, LAMP2 for lysosomes). Western blots of purified 30 fractions from cell lysates enriched for different intracellular membrane bound organelles (e.g., lysosomes, synaptosomes, Golgi) may also be employed. In addition, the relative orientation of

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different domains of the CalDAG-GEFs and/or cAMP-GEFs across cellular domains may be assayed using, for example, electron microscopy and antibodies raised to those domains.

9. Screening and Diagnostics for CalDAG-GEF- or cAMP-GEF-associated disorders

A. General Diagnostic Methods

5       The CalDAG-GEF or cAMP-GEF genes and gene products, as well as the CalDAG-GEF or cAMP-GEF-derived probes, primers and antibodies, disclosed or otherwise enabled herein, are useful in the screening for carriers of alleles associated with CalDAG-GEF- or cAMP-GEF-associated disorders. Individuals at risk for such a disorder or individuals not previously known to be at risk, may be routinely screened using probes to detect the presence of a mutant CalDAG-  
10      GEF or cAMP-GEF gene or protein by a variety of techniques. Diagnosis of inherited cases of these diseases can be accomplished by methods based upon the nucleic acids (including genomic and mRNA/cDNA sequences), proteins, and/or antibodies disclosed and enabled herein, including functional assays designed to detect increases or decreases of the normal CalDAG-GEF or cAMP-GEF activity and/or the presence of specific new activities conferred by the mutant  
15      CalDAG-GEFs or cAMP-GEFs. Preferably, the methods and products are based upon the human CalDAG-GEF or cAMP-GEF nucleic acids, proteins or antibodies, as disclosed or otherwise enabled herein. For brevity of exposition, but without limiting the scope of the invention, the following description will focus upon uses of the human homologues of CalDAG-GEF and cAMP-GEF. It will be understood, however, that homologous sequences from other species, including those disclosed herein, will be equivalent for many purposes.  
20

B. Protein Based Screens and Diagnostics

When a diagnostic assay is to be based upon CalDAG-GEF or cAMP-GEF proteins, a variety of approaches are possible. For example, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will  
25      be particularly useful in identifying mutants in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

30      C. Nucleic Acid Based Screens and Diagnostics

When the diagnostic assay is to be based upon nucleic acids from a sample, the assay may be based upon mRNA, cDNA or genomic DNA. Whether mRNA, cDNA, or genomic DNA is

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assayed, standard methods well known in the art may be used to detect the presence of a particular sequence either *in situ* or *in vitro* (See, e.g., Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed. (1989)).

(1) Appropriate Probes and Primers

- 5 Whether for hybridization, RNase protection, ligase-mediated detection, PCR amplification or any other standards methods described herein and well known in the art, a variety of subsequences of the CalDAG-GEF and/or cAMP-GEF sequences disclosed or otherwise enabled herein will be useful as probes and/or primers. These sequences or subsequences will include both normal CalDAG-GEF or cAMP-GEF sequences and deleterious 10 mutant sequences. In general, useful sequences will include at least 8-10, more preferably 10-15, and most preferably 15-25 consecutive nucleotides from the CalDAG-GEF or cAMP-GEF introns, exons or intron/exon boundaries. In another embodiment, useful sequences include at least 25-500 consecutive nucleotides. Depending upon the target sequence, the specificity required, and future technological developments, shorter sequences may also have utility.
- 15 Therefore, any CalDAG-GEF or cAMP-GEF derived sequence which is employed to isolate, clone, amplify, identify or otherwise manipulate a CalDAG-GEF or cAMP-GEF sequence may be regarded as an appropriate probe or primer.

(2) Hybridization Screening

- For *in situ* detection of a normal or mutant CalDAG-GEF, cAMP-GEF or other CalDAG-20 GEF and/or cAMP-GEF-associated nucleic acid sequence, a sample of tissue may be prepared by standard techniques and then contacted with one or more of the above-described probes, preferably one which is labeled to facilitate detection, and an assay for nucleic acid hybridization is conducted under stringent conditions which permit hybridization only between the probe and highly or perfectly complementary sequences.

25 (3) Restriction Mapping

- Sequence alterations may also create or destroy fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by electrophoresis and visualization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or decrease of corresponding restriction fragment 30 numbers. Such restriction fragment length polymorphism analysis (RFLP), or restriction mapping, may be employed with genomic DNA, mRNA or cDNA. The CalDAG-GEF or cAMP-GEF sequences may be amplified by PCR using the above-described primers prior to

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restriction, in which case the lengths of the PCR products may indicate the presence or absence of particular restriction sites, and/or may be subjected to restriction after amplification. The CalDAG-GEF or cAMP-GEF fragments may be visualized by any convenient means (e.g., under UV light in the presence of ethidium bromide).

5           (4) PCR Mapping

In another series of embodiments, a single base substitution mutation may be detected based on differential PCR product length or production in PCR. Thus, primers which span mutant sites or which, preferably, have 3' termini at mutation sites, may be employed to amplify a sample of genomic DNA, mRNA or cDNA from a subject. A mismatch at a mutational site may 10 be expected to alter the ability of the normal or mutant primers to promote the polymerase reaction and, thereby, result in product profiles which differ between normal subjects and heterozygous and/or homozygous CalDAG-GEF or cAMP-GEF mutants.

(5) Electrophoretic Mobility

Genetic testing based on DNA sequence differences also may be achieved by detection of 15 alterations in electrophoretic mobility of DNA, mRNA or cDNA fragments in gels. Small sequence deletions and insertions, for example, can be visualized by high resolution gel electrophoresis of single or double stranded DNA, or as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis.

(6) Chemical Cleavage of Mismatches

Mutations in the CalDAG-GEFs or cAMP-GEFs may also be detected by employing the 20 chemical cleavage of mismatch (CCM) method (*See, e.g., Saleeba et al., METHODS IN ENZYMOLOGY, 217: 286-295 (1993)*). In this technique, probes (up to ~ 1 kb) may be mixed with a sample of genomic DNA, cDNA or mRNA obtained from a subject. The sample and probes are mixed and subjected to conditions which allow for heteroduplex formation (if any).  
25 Preferably, both the probe and sample nucleic acids are double-stranded, or the probe and sample may be PCR amplified together, to ensure creation of all possible mismatch heteroduplexes. Mismatched T residues are reactive to osmium tetroxide and mismatched C residues are reactive to hydroxylamine. Because each mismatched A will be accompanied by a mismatched T, and each mismatched G will be accompanied by a mismatched C, any nucleotide differences between 30 the probe and sample (including small insertions or deletions) will lead to the formation of at least one reactive heteroduplex. After treatment with osmium tetroxide and/or hydroxylamine to modify any mismatch sites, the mixture is subjected to chemical cleavage at any modified

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mismatch sites by, for example, reaction with piperidine. The mixture may then be analyzed by standard techniques such as gel electrophoresis to detect cleavage products which would indicate mismatches between the probe and sample.

(7) Other Methods

5 Various other methods of detecting CalDAG-GEF or cAMP-GEF mutations, based upon the CalDAG-GEF or cAMP-GEF sequences disclosed and otherwise enabled herein, will be apparent to those of ordinary skill in the art. Any of these may be employed in accordance with the present invention. These include, but are not limited to, nuclease protection assays (S1 or ligase-mediated), ligated PCR, denaturing gradient gel electrophoresis (DGGE; *see, e.g.*, Fischer  
10 et al., 80 PROC. NAT'L ACAD. SCI (USA), 1578-83 (1983)), restriction endonuclease fingerprinting combined with SSCP (REF-SSCP; *see, e.g.*, Liu et al., 18 BIOTECHNIQUES 470-79 (1995)), and the like.

D. Other Screens and Diagnostics

Diagnosis also can be made by observation of alterations in CalDAG-GEF or cAMP-GEF  
15 transcription, translation, and post-translational modification and processing as well as alterations in the intracellular and extracellular trafficking of CalDAG-GEF or cAMP-GEF gene products in the brain and peripheral cells. Such changes will include alterations in the amount of CalDAG-GEF or cAMP-GEF messenger RNA and/or protein, alteration in phosphorylation state, abnormal intracellular location/distribution, abnormal extracellular distribution, etc. Such assays  
20 will include: Northern Blots (with CalDAG-GEF or cAMP-GEF-specific and non-specific nucleotide probes), Western blots and enzyme-linked immunosorbent assays (ELISA) (with antibodies raised specifically to a CalDAG-GEF or a cAMP-GEF functional domain, including various post-translational modification states).

E. Screening and Diagnostic Kits

25 In accordance with the present invention, diagnostic kits are also provided which will include the reagents necessary for the above-described diagnostic screens. For example, kits may be provided which include antibodies or sets of antibodies which are specific to one or more mutant epitopes. These antibodies may, in particular, be labeled by any of the standard means which facilitate visualization of binding. Alternatively, kits may be provided in which  
30 oligonucleotide probes or PCR primers, as described above, are present for the detection and/or amplification of mutant CalDAG-GEF, cAMP-GEF or other CalDAG-GEF and/or cAMP-GEF-associated nucleotide sequences. Again, such probes may be labeled for easier detection of

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specific hybridization. As appropriate to the various diagnostic embodiments described above, the oligonucleotide probes or antibodies in such kits may be immobilized to substrates and appropriate controls may be provided.

#### 10. Methods of Treatment

5       The present invention now provides a basis for therapeutic intervention in diseases which are associated to the CalDAG-GEFs or cAMP-GEFs in that they are caused, prevented, exacerbated, or alleviated, or which may be caused, prevented, exacerbated, or alleviated, by the either normal or mutant CalDAG-GEFs or cAMP-GEFs. In considering the various therapies described below, it is understood that such therapies may be targeted at tissue other than the brain  
10 where CalDAG-GEF or cAMP-GEF are also expressed.

Therapies to treat CalDAG-GEF and/or cAMP-GEF-associated diseases may be based upon (1) administration of normal CalDAG-GEF or cAMP-GEF proteins, (2) gene therapy with normal CalDAG-GEF or cAMP-GEF genes to compensate for or replace the mutant genes, (3) gene therapy based upon antisense sequences to mutant CalDAG-GEF or cAMP-GEF genes or  
15 which "knock-out" the mutant genes, (4) gene therapy based upon sequences which encode a protein which blocks or corrects the deleterious effects of CalDAG-GEF or cAMP-GEF mutants, (5) immunotherapy based upon antibodies to normal and/or mutant CalDAG-GEF or cAMP-GEF proteins, or (6) small molecules (drugs) which alter CalDAG-GEF or cAMP-GEF expression,  
block abnormal interactions between mutant forms of CalDAG-GEF or cAMP-GEF and other  
20 proteins or ligands, or which otherwise block the aberrant function of mutant CalDAG-GEF or cAMP-GEF proteins by altering the structure of the mutant proteins, by enhancing their metabolic clearance, or by inhibiting their function.

##### A. Protein Therapy

Treatment of CalDAG-GEF and/or cAMP-GEF-associated disorders, or disorders  
25 resulting from CalDAG-GEF and/or cAMP-GEF mutations, may be performed by providing an excess of inactive mutant protein to decrease the effect of the normal function of the protein, or by providing an excess of normal protein to reduce the effect of any aberrant function of the mutant protein, by replacing a mutant protein with normal protein, or by modulating the function of the mutant protein.

##### 30       B. Gene Therapy

In one series of embodiments, gene therapy may be employed in which normal or mutant copies of the CalDAG-GEF gene or the cAMP-GEF gene are introduced into patients to code

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successfully for normal or mutant protein in one or more different affected cell types. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Thus, it is preferred that the recombinant gene be operably joined to a strong promoter so as to provide a high level of expression which will compensate for, or out-compete, the naturally-occurring proteins. As noted above, the recombinant construct may contain endogenous or exogenous regulatory elements, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

In another series of embodiments, gene therapy may be employed to replace the naturally-occurring gene by homologous recombination with a recombinant construct. The recombinant construct may contain a normal or a mutant copy of the targeted CalDAG-GEF and/or cAMP-GEF gene, in which case the defect is corrected *in situ*, or may contain a "knock-out" construct which introduces a stop codon, missense mutation, or deletion which abolished function of the mutant gene. It should be noted in this respect that such a construct may knock-out both the normal and mutant copies of the targeted CalDAG-GEF and/or cAMP-GEF gene in a heterozygous individual, but the total loss of CalDAG-GEF and/or cAMP-GEF gene function may be less deleterious to the individual than continued progression of the disease state.

In another series of embodiments, antisense gene therapy may be employed. The antisense therapy is based on the fact that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA or DNA and a complementary antisense species. The formation of a hybrid duplex may then interfere with the transcription of the gene and/or the processing, transport, translation and/or stability of the target CalDAG-GEF and/or cAMP-GEF mRNA. Antisense strategies may use a variety of approaches including the administration of antisense oligonucleotides or antisense oligonucleotide analogs (*e.g.*, analogs with phosphorothioate backbones) or transfection with antisense RNA expression vectors. Again, such vectors may include exogenous or endogenous regulatory regions, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

In another series of embodiments, gene therapy may be used to introduce a recombinant construct encoding a protein or peptide which blocks or otherwise corrects the aberrant function caused by a naturally-occurring CalDAG-GEF and/or cAMP-GEF gene. In one embodiment, the recombinant gene may encode a peptide which corresponds to a mutant domain of a CalDAG-GEF and/or cAMP-GEF which has been found to abnormally interact with another cell protein or other cell ligand. Alternatively, the portion of a protein which interacts with a mutant, but not a

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normal, CalDAG-GEF and/or cAMP-GEF may be encoded and expressed by a recombinant construct in order to compete with, and thereby inhibit or block, the aberrant interaction.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however  
5 must be able to divide and the expression of the levels of normal protein should be high. The full length CalDAG-GEF or cAMP-GEF genes, subsequences encoding functional domains of the CalDAG-GEFs or cAMP-GEFs, or any of the other therapeutic peptides described above, can be cloned into a retroviral vector and driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for the target cell type of interest. Other viral  
10 vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr virus.

C. Immunotherapy

Antibodies may be raised to a mutant CalDAG-GEF or cAMP-GEF protein (or a portion thereof) and be administered to a patient to bind or block the mutant protein and prevent its deleterious effects. Alternatively, antibodies may be raised to specific complexes between  
15 mutant or wild-type CalDAG-GEF or cAMP-GEF and their interaction partners.

A further approach is to stimulate endogenous antibody production to the desired antigen. An immunogenic composition may be prepared as injectables, as liquid solutions or emulsions. The CalDAG-GEF or cAMP-GEF protein or other antigen may be mixed with pharmaceutically  
20 acceptable excipients compatible with the protein. Such excipients may include water, saline, dextrose, glycerol, ethanol and combinations thereof. The immunogenic composition and vaccine may further contain auxiliary substances such as emulsifying agents or adjuvants to enhance effectiveness. Immunogenic compositions and vaccines may be administered parenterally by injection subcutaneously or intramuscularly.

25 The immunogenic preparations and vaccines are administered in such amount as will be therapeutically effective, protective and immunogenic. Dosage depends on the route of administration and will vary according to the size of the host.

D. Small Molecule Therapeutics

As described and enabled herein, the present invention provides for a number of methods  
30 of identifying small molecules or other compounds which may be useful in the treatment of CalDAG-GEF- or cAMP-GEF-associated disorders. Thus, for example, the present invention provides for methods of identifying CalDAG-GEF or cAMP-GEF binding proteins and, in

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particular, methods for identifying proteins or other cell components which bind to or otherwise interact with mutant CalDAG-GEFs or cAMP-GEFs but not with the normal CalDAG-GEFs or cAMP-GEFs. The invention also provides for methods of identifying small molecules which can be used to disrupt undesired interactions between CalDAG-GEFs or cAMP-GEFs and other 5 proteins or other cell components.

By identifying these proteins and analyzing these interactions, it is possible to screen for or design compounds which counteract or prevent the interaction, thereby, providing treatment for abnormal interactions. Therapies can be designed to modulate these interactions and thereby, to modulate CalDAG-GEF- or cAMP-GEF-associated disorders. The potential efficacy of these 10 therapies can be tested by analyzing the affinity and function of these interactions after exposure to the therapeutic agent by standard pharmacokinetic measurements of affinity (e.g., Kd, Vmax) using synthetic peptides or recombinant proteins corresponding to functional domains of the CalDAG-GEF gene, the cAMP-GEF gene or other CalDAG-GEF and/or cAMP-GEF homologues. Another method for assaying the effect of any interactions involving functional 15 domains is to monitor changes in the intracellular trafficking and post-translational modification of the relevant genes by *in situ* hybridization, immunohistochemistry, Western blotting and metabolic pulse-chase labeling studies in the presence of, and in the absence of, the therapeutic agents. A further method is to monitor the effects of "downstream" events including changes in second messenger events, e.g., cAMP, intracellular Ca<sup>2+</sup>, protein kinase activities, etc.

20 The effect of potential therapeutic agents in cell lines and whole animals can be monitored by monitoring transcription, translation, and post-translational modification of the CalDAG-GEF and/or cAMP-GEF proteins. Methods for these studies include Western and Northern blots, immunoprecipitation after metabolic labelling (pulse-chase) with radio-labelled methionine and ATP, and immunohistochemistry. The effect of these agents can also be 25 monitored using studies which examine the relative binding affinities and relative amounts of CalDAG-GEF or cAMP-GEF proteins involved in interactions with Rap1A, using either standard binding affinity assays or co-precipitation and Western blots using antibodies to Rap1A, CalDAG-GEF, cAMP-GEF, or other CalDAG-GEF and/or cAMP-GEF homologues.

Therapy using antisense oligonucleotides to block the expression of the mutant CalDAG- 30 GEF gene or the mutant cAMP-GEF gene, co-ordinated with gene replacement with normal CalDAG-GEF or cAMP-GEF gene can also be applied using standard techniques of either gene therapy or protein replacement therapy.

**V. Examples****Example 1: Isolation and characterization of CalDAG-GEF.**

Human full-length CalDAG-GEFI cDNAs were isolated from a human frontal cortex λZAPII cDNA library (Stratagene) and a U937 λZAPII cDNA library. Mouse full-length 5 CalDAG-GEFI was identified in the mouse EST database (GenBank accession number: W71787). Rat full-length CalDAG-GEFII cDNA was isolated from a rat whole brain λZAPII cDNA library by using human CalDAG-GEFII as a probe. Mouse ESTs identified through BLAST searches were purchased from Genome Systems Inc. (St. Louis, MO).

CalDAG-GEFI encodes an approximately 69-kD protein (Fig. 2D) that displays in its 10 amino terminal region a GEF domain that is highly homologous to Ras-superfamily GEFs (Fig. 2A-2D). Multiple alignment analysis shows that genes of the CalDAG-GEF family form a cluster within the Ras-GEF superfamily distinct from Ras GEFs such as Sos1 and rRas-GEF (Fig. 2B). The region downstream of the GEF domain contains two tandem repeats of EF-hand Ca<sup>2+</sup> binding motifs (Figs. 2A, 2E). The carboxy-terminal region displays a typical 15 diacylglycerol/phorbol ester-binding domain, which is present in most PKC family proteins (Fig. 2A, 2F). Multiple sequence alignments and phylogenetic tree analysis were carried out with the LASERGENE Software Package (DNASTAR Inc.). Abbreviations and GenBank accession numbers of the protein sequences used in Figure 2 are as follows: C3G: 474982, mCdc25: 882120, rRas-GRF: 57665, hSos1 (human son-of-sevenless 1): 476780, BUD5: 171141, 20 hCalmodulin: 115512, hCalbindin D28k: 227666, hCalcineurin B: 105504, hParvalbumin a: 131100, hTroponin C: 136043, hPKCa: 125549, hPKCb1: 125538, hPKCg: 462455.

To determine the small G protein target of CalDAG-GEFI, guanine nucleotide exchange activity *in vivo* was analyzed using intact 293T cells cotransfected with a eukaryotic expression construct of mouse CalDAG-GEFI and GST-tagged Ras family proteins. Full-length mouse 25 CalDAG-GEFI cDNA inserted into a pCMV-SPORT expression vector with a carboxy-terminal FLAG epitope was used for transfection. A PCR-amplified fragment of rat CalDAG-GEFII was subcloned into a pCAGGS expression vector with the addition of His<sub>6</sub>-tag at its amino-terminus, resulting in pCAGGS-His-CalDAGII. pEBG-Krev1 that expresses Rap1A was used as a fusion protein to glutathione S-transferase (GST) in mammalian cells, as described in Gotoh et al., 15 Mol. Cell Biol. 6746-53 (1995), pEBG-R-Ras, other vectors for Ras-family proteins obtained by inserting PCR-amplified cDNAs into pEBG expression vector, pCAGGS-C3G and pCAGGS- 30 MSos1, and pCEV-H-RasV12. CalDAG-GEFI transfection produced a dramatic increase in

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GTP-bound Rap1A compared to the control but showed no or minimal activation of H-Ras, R-Ras, or Ral A. The increase in GTP-bound Rap1A was augmented in the presence of either the Ca<sup>2+</sup> ionophore, A23187, or the phorbol ester, phorbol-12-myristate-13-acetate (TPA). Further, A23187 and TPA had additive effects when administered together.

5 To determine the effect of CalDAG-GEFI on the Erk/MAP kinase cascade, Elk1 activation was measured in 293T cells transfected with CalDAG-GEFI or constitutively active H-Ras (RasV12), or both. 293T cells were transfected by SuperFect (Qiagen) as described in Gotoh, *supra*, with expression vectors for GST-tagged Ras family proteins and with those for various GEFs. Cells were labeled 24 hours after transfection with <sup>32</sup>P, for 2 hr. In some 10 experiments, cells were stimulated with either 10 µM A23187 or 1 µM phorbol-12-myristate-13-acetate (TPA) for 3 min. GST-tagged Ras family proteins were collected from cell lysates with glutathione Sepharose. Guanine nucleotides bound to Ras family proteins were separated by thin layer chromatography (TLC). Activation of Elk1 was examined by the PathDetect Elk1 transreporting system (Stratagene). 293T cells were transfected with pFR-Luc and pFA-Elk1 15 with various expression vectors, and light output was detected and analyzed by the use of LAS1000 film. CalDAG-GEFI reduced RasV12 activation of Elk1 by approximately 4-fold and did not itself activate Elk1. Thus, CalDAG-GEFI strongly inhibits Ras-dependent stimulation of the Erk/MAP kinase cascade.

Northern analysis showed that human CalDAG-GEFI is expressed strongly in the brain 20 and that CalDAG-GEFI mRNA is strikingly enriched in the striatum. Probes used included human CalDAG-GEFI: 729-bp EcoRI fragment, human CalDAG-GEFII: 584-bp SacI and HindIII fragment, rat CalDAG-GEFI: 439-bp fragment of EST clone RBC565 (GenBank accession number: C06861, and rat CalDAG-GEFII: 508-bp PCR amplified and subcloned fragment (nucleic acids 2541 to 3048 of SEQ ID NO:5). *In situ* hybridization of sections from 25 the adult rat brain confirmed these restricted distribution patterns. Intense signal was present in the striatum (caudoputamen) and the ventral striatum (nucleus accumbens, olfactory tubercle). There was weaker signal in the olfactory bulb.

A series of monoclonal antibodies against the carboxy-terminal half of mouse CalDAG-GEFI were raised. His<sub>6</sub>-tagged mouse CalDAG-GEFI polypeptide (amino acids 349 to 608 of 30 SEQ ID NO:1) was expressed in bacteria, purified over Ni<sup>2+</sup>-nitrilotriacetic acid-agarose resin, and then used to immunize BALB/c mice. The resultant polyclonal antiserum was monitored by ELISA, Western blot, immunoprecipitation, and immunofluorescence assays on CalDAG-GEFI-

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transfected COS-7 cells. Hybridomas were generated by PEG (polyethylene glycol)-mediated fusion of donor splenocytes to the SP2/O cell line. Positive hybridoma cell lines were identified by screening in the assays described above, and purified by limiting dilution and single-cell cloning. Three hybridoma cell lines against mouse CalDAG-GEFI (mAbs 18B11, 2D9, and 5 18A7), in addition to the polyclonal fusion serum, were identified. Western analysis showed that mAbs 18B11 and 2D9 were specific for CalDAG-GEFI. Lightly post-fixed, cryostat-cut 10 µm thick sections were immunostained by the ABC (Vectastain kit) method for CalDAG-GEFI with mAbs 18B11 and 2D9 and the polyclonal fusion serum, for tyrosine hydroxylase (TH) with monoclonal antibodies from INCSTAR, and for µ opioid receptor with polyclonal antiserum. 10 Immunohistochemistry with mAb 18B11 showed a striking basal ganglia-enriched distribution pattern in sections of adult rat brain, with significant but weaker activity elsewhere. CalDAG-GEFI immunoreactivity marked the entire pathway from the striatal matrix compartment to the pallidum and substantia nigra pars reticulata, where very intense CalDAG-GEFI staining was present. Thus, CalDAG-GEFI is synthesized in striatal projection neurons and is transported to 15 striatopallidal and striatonigral terminals.

To confirm that CalDAG-GEFI is synthesized in striatal projection neurons and transported to striatopallidal and striatonigral terminals in rats, intrastriatal injections of ibotenic acid (20 µg/µl, 1.5 µl per site, 5 day survival) were made unilaterally at 2 sites in the mid-lateral caudoputamen, with contralateral vehicle control injections were made. In other rats, unilateral 20 subthalamic knife-cuts were made at an anteroposterior level between the entopeduncular nucleus and substantia nigra to sever the striatonigral efferents (1 and 3 days survivals), with control contralateral thalamic knife-cuts. These procedures all reduced CalDAG-GEFI staining in the substantia nigra. *In situ* hybridization was performed according to Simmons et al, 12 J. Histotechnol. 169-181 (1989). A 439bp rat EST clone RBC565 (98.4% identical to mouse 25 CalDAG-GEFI nucleic acids 1777 to 2216 of SEQ ID NO:1) was isolated by BLAST search and used for making RNA probes with <sup>32</sup>P-labeled UTP (2,000 Ci/mmol, NEN, 1 Ci = 37 GBq) and T3 and T7 RNA polymerase. Brains were processed as above for CalDAG-GEFI and TH immunostaining. Thus, CalDAG-GEFI is a protein transported in striatal axons to their terminals. The terminal localization of CalDAG-GEFI was confirmed in subcellular 30 fractionation experiments on dissected samples from the rat ventral midbrain, in which Western analysis showed the presence of CalDAG-GEFI in cytosol and in membrane fractions, including synaptosomes.

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Because of the similarity of the GEF domains of CalDAG-GEFI and CalDAG-GEFII, the substrate specificity of CalDAG-GEFII with the same 293T cell assay system used for CalDAG-GEFI was examined. It was confirmed that CalDAG-GEFII activates Ras, and further shown that it activates H-Ras and R-Ras, but not Ral A or Rap1A. H-Ras activation was enhanced by 5 A23187 and TPA. Moreover, CalDAG-GEFII, unlike CalDAG-GEFI, increased the transcriptional activity of Elk1 downstream to Erk/MAP kinase. Thus, in the 293T system, CalDAG-GEFI and CalDAG-GEFII target different Ras-superfamily small G proteins and have opposite effects on the MAP kinase cascade. Northern analysis further showed contrasting brain expression for CalDAG-GEFII, with highest expression being in the cerebellum, cerebral cortex, 10 and amygdala, and low expression occurring in the striatum. Both genes are also expressed in hematopoietic organs in both human and rat.

Rap signaling is important in regulating basal ganglia output in response to  $\text{Ca}^{2+}$  and DAG. Corticostriatal inputs can activate the MAP kinase cascade in striatal projection neurons (Sgambat et al., 18 J. Neurosci. 214-26 (1993)) and phosphoinositide (PI) signaling is strongly 15 represented in these pathways (Fotuhi et al., 13 J. Neurosci. 3300-08 (1993)). Moreover, a number of receptor systems in the striatum and its striatonigral/striatopallidal pathways are linked to  $\text{Ca}^{2+}$  and PI signaling, notably including NMDA and metabotropic glutamate receptors, D<sub>2</sub>-class dopamine receptors, and tachykinin receptors (Fiorillo et al., 394 Nature 78-82 (1998)). A previously unrecognized signaling target for some of these systems is likely to be Rap1, via 20 CalDAG-GEFI. In addition, CalDAG-GEFI has a synaptic function as demonstrated by the heavy accumulation of CalDAG-GEFI in the target nuclei of striatal outputs and the localization of Rap1 in synaptosomes and synaptic vesicles. The particular basal ganglia projection systems are enriched in CalDAG-GEFI and are differentially vulnerable to neurodegeneration in Huntington's disease.

25 Rap and Ras functions can be regulated coordinately or disjunctively by  $\text{Ca}^{2+}$  and DAG in the brain and hematopoietic organs, depending on the relative expression of CalDAG-GEFI and CalDAG-GEFII. In neurons, Ras/MAP kinase signaling has been directly implicated in synaptic transmission and the neuroplasticity underlying learning and memory. Different CalDAG-GEFI and CalDAG-GEFII expression patterns in the brain influence region-specific neuroplasticity 30 mediated by  $\text{Ca}^{2+}$  and DAG signaling pathways. The presence of CalDAG-GEFI and CalDAG-GEFII in the hematopoietic system demonstrates the direct input of  $\text{Ca}^{2+}$  and DAG to Ras/Rap regulation of normal growth and differentiation as well as malignant transformation.

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Example 2: Isolation and characterization of cAMP-GEFs.

cAMP-GEFI and cAMP-GEFII have similar domain structures, with a cAMP binding domain at the amino terminus and a GEF domain at the carboxy terminus separated by a link region (LR) (Fig. 3A). These mammalian proteins show strong structural homology to a 5 predicted open reading frame (T20G5.5) in *C. elegans* cAMP-GEF (cel cAMP-GEF) (Fig. 3A). The cAMP binding domains of the cAMP-GEF family proteins form a distinct group within the cyclic nucleotide-binding protein superfamily and show the closest similarity to the B domains of PKA regulatory subunits (Fig. 3B). A PR(A/T)AT motif in the cAMP binding pocket is also conserved in the cAMP-GEF proteins (Fig. 3E). The first alanine of this motif confers cAMP 10 (alanine) as opposed to cGMP (threonine) binding specificity. All of the cAMP-GEF family members have alanine at this position, and therefore bind cAMP rather than cGMP.

The GEF domains of the cAMP-GEFs show high homology to those of Ras-GEF family proteins, but form an independent cluster distinct from Ras GEFs such as mCdc25, hSos1, and rRas-GRF (Fig. 3, C and D). The three structurally conserved regions specific to Ras-GEF 15 family proteins (SCR1, SCR2, and SCR3) are present in all of the cAMP-GEF proteins (Fig. 3D). Multiple sequence alignments and phylogenetic tree analyses were carried out with LASERGENE (DNASTAR Inc.). Abbreviations and GenBank accession numbers of the protein sequences used in this figure: hPKARI $\alpha$  (human cAMP-dependent protein kinase regulatory subunit type I-alpha): 125193, hPKARI $\beta$ : 1346362, hPKARII $\alpha$ : 125198, hPKARII $\beta$ : 400115, 20 hPKG $\alpha$  (human cGMP-dependent protein kinase type I-alpha): 1255602, hPKG $\beta$ : 125379, hPKGII: 1906312, hCalDAG-GEFI: U71870, hCalDAG-GEFII: AF081195, C3G: 474982, hSos1 (human son-of-sevenless 1): 476780, mCdc25: 882120, rRas-GRF: 57665, BUDS: 171141.

In order to identify the small G protein substrate for cAMP-GEFI and II and the mode of 25 cAMP regulation of GEF activity conferred by these proteins, the effects of cAMP-GEFI and cAMP-GEFII expression were analyzed in 293T cells on the ratio of GTP to GDP bound to different Ras family small G proteins in the presence or absence of forskolin and IBMX. Under basal conditions, in the absence of forskolin and IBMX, only Rap1 was activated significantly. In the presence of forskolin and IBMX, both cAMP-GEFI and II strongly and selectively 30 activated Rap1A, but did not activate H-Ras, R-Ras or RalA. The effects of forskolin/IBMX treatment on cAMP-GEFI and II were dose-dependent with EC<sub>50</sub> values of 1.8  $\mu$ M and 0.3  $\mu$ M, respectively. Forskolin/IBMX treatment given alone had no effect.

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A time-course analysis of the activation of Rap1A by forskolin/IBMX in cAMP-GEFI transfectants showed that the activation began within 10 sec, reached a maximum at 5 min, and continued for at least 60 min. Thus, cAMP-GEFI has a direct effect on Rap1A rather than secondary effects mediated by other Ras-superfamily GEFs. In addition, Sp-cAMPS, an 5 analogue of cAMP, activated Rap1A at levels similar to those induced by forskolin/IBMX. Thus, cAMP has the capacity to activate the GEF domain of cAMP-GEFI.

Mutational analyses with cAMP-GEFI was performed to examine whether its cAMP-binding domain is required for the activation of Rap1A. In contrast to wild type cAMP-GEFI, a deletion mutant lacking a cAMP binding domain (pcDNA-rcAMP-GEFI:DcAMP(528) and 10 (595)) did not activate Rap1A with or without forskolin/IBMX treatment. Mutants with a single amino acid substitution at the cAMP binding pocket (pcDNA-rcAMP-GEFI:R(279)K) responded minimally to forskolin/IBMX treatment. Thus, the cAMP binding domain of cAMP-GEFI is necessary for its cAMP-dependent activation of Rap1A.

To assess further the cAMP binding capacity of cAMP-GEFI, a cAMP agarose affinity 15 bead binding assay was performed. *In vitro* translated, radiolabeled cAMP-GEFI showed selective binding to the beads that was competed by excess amounts of either cAMP or 8-Br-cAMP. cAMP-GEF protein can bind cAMP and that this binding can activate Rap1A.

cAMP-dependent activation of Rap1 has previously been ascribed to the phosphorylation of Rap1A by PKA, which raises its affinity to smgGDS, a GEF with broad substrate specificity. 20 However, at least in the 293T cell assay system, an increase of GTP-bound Rap1A in response to increasing cAMP levels with forskolin or treatment with the cAMP analogue, Sp-cAMPS was not detected in the absence of cAMP-GEFs. In addition, even in the presence of H-89, a potent and selective inhibitor of PKA, cAMP-GEFI and II could still activate Rap1A. The activation of Rap1A induced by cAMP-GEFI and II is independent of the PKA pathway.

Intracellular cAMP has been shown to interact directly with ion channels, but the vast 25 majority of cAMP-mediated effects in eukaryotes have been considered as sequels to cAMP binding by the regulatory subunits of the PKA tetramer. The diversity of physiological effects produced by cAMP have been attributed to the fact that, as a kinase, PKA has a large range of molecular targets. Reported herein are novel cAMP binding proteins that directly link the cAMP 30 second messenger system to Ras superfamily signaling pathways and that appear selectively to target Rap.

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- cAMP can exert profound cell-type specific effects on cell growth and differentiation and that cAMP is capable of inhibiting or stimulating the Ras/mitogen-activated protein (MAP) kinase/Erk pathway. The inhibition can occur at the initial translocation step by which Ras activates Raf, whereas activation of Rap1 is thought to occur through phosphorylation by PKA.
- 5 Activation of Rap1 has been suggested to be part of a switch mechanism determining whether growth or differentiation occurs in response to nerve growth factor (NGF). cAMP-GEFs directly couple cAMP to Rap1, itself discovered as a negative regulator of Ras but suspected of having independent functions as well. Thus, different levels of cAMP-GEF expression confer cell-type specific regulation of Ras superfamily signaling systems.

10 The genes also exhibit developmentally regulated expression in the septum, medial thalamus and habenula, key structures in the limbic system variously linked to brain reward circuits, addiction and schizophrenia. Thus, cAMP-GEFs, in addition to PKA, underlie some of the neuronal functions of cAMP.

Example 3. Northern hybridization demonstrating the expression of CalDAG-GEFI and  
15 CalDAG-GEFII protein mRNAs in a variety of tissues.

Total cytoplasmic RNA was isolated from various human tissue samples including amygdala, cerebellum, corpus callosum, caudate nucleus, cortex, frontal lobe, hippocampus, kidney, liver, lung, medulla oblongata, occipital pole, putamen, spinal cord, substantia nigra, subthalamic nucleus, thalamus, and temporal lobe, obtained from surgical pathology using  
20 standard procedures such as CsCl purification. The RNA was then electrophoresed on a formaldehyde gel to permit size fractionation. The nitrocellulose membrane was prepared and the RNA was then transferred onto the membrane. <sup>32</sup>P-labeled cDNA probes were prepared and added to the membrane in order for hybridization between the probe and the RNA to occur. After washing, the membrane was wrapped in plastic film and placed into imaging cassettes containing  
25 X-ray film. The autoradiographs were then allowed to develop for one to several days. Sizing was established by comparison to standard RNA markers. These northern blots demonstrated that the CalDAG-GEF genes are strongly expressed in the brain. Weaker hybridization was detectable elsewhere.

Example 4. Northern hybridization demonstrating the expression of cAMP-GEFI and cAMP-  
30 GEFII protein mRNAs in a variety of tissues.

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Northern hybridization analysis was performed as in Example 3 to detect the expression of the cAMP-GEFI and cAMP-GEFII genes in a variety of human tissues. The tissues analyzed included adrenal gland, amygdala, bone marrow, cerebellum, corpus callosum, caudate nucleus, colon (mucosal lining), caudputamen, cortex, frontal lobe, hippocampus, habenula, heart, kidney, liver, lung, lymph node, medulla oblongata, occipital pole, olfactory bulb, ovary, pons, pancreas, putamen, septum, small intestines, skeletal muscle, spinal cord, spleen, stomach, substantia nigra, subthalamic nucleus, testis, thalamus, temporal lobe, thymus, trachea, and thyroid.

A striking contrast in the expression patterns of human cAMP-GEFI and II was observed by Northern analysis. Human cAMP-GEFI is widely expressed, with highest levels appearing in kidney, spleen, thyroid, heart, and pancreas. Human cAMP-GEFII shows a remarkably selective enrichment in the brain and the adrenal glands. Both genes were developmentally regulated. The expression patterns of the two genes in the nervous system also differ, with cAMP-GEFI having a wider expression than cAMP-GEFII. These region-specific neuronal expression patterns were confirmed in *in situ* hybridization experiments. cAMP-GEFI mRNA is expressed broadly at low levels in the adult brain, but it is strongly and selectively expressed in parts of the neonatal brain, including the septum and the thalamus. By contrast, cAMP-GEFII is strongly expressed in the mature as well as the developing brain. Notable are the high levels of cAMP-GEFII mRNA in the cerebral cortex, the hippocampus (especially CA3 and dentate gyrus), the habenula and the cerebellum. Genes of the cAMP-GEF family have widespread influence on cAMP functions in bodily organs and also region-specific functions in the brain.

Example 5. Isolation of CalDAG-GEF or cAMP-GEF binding proteins by yeast two-hybrid system.

To identify proteins interacting with the CalDAG-GEF or cAMP-GEF proteins, a yeast expression plasmid vector (pAS2-1, Clontech) is generated by ligating an in-frame partial cDNA sequence encoding either residues of the CalDAG-GEF protein or residues of the cAMP-GEF protein into the EcoRI and BamHI sites of the vector. The resultant fusion protein contains the GAL4 DNA binding domain coupled in-frame either to residues of the CalDAG-GEF protein or to residues of the cAMP-GEF protein. These expression plasmids are co-transformed, along with purified plasmid DNA from the human brain cDNA:pACT library, into yeast using the protocols of the Clontech Matchmaker yeast-two-hybrid kit (Clontech). Yeast clones bearing human brain cDNAs which interact with the CalDAG-GEF or cAMP-GEF fragments are selected by HIS resistance and  $\beta$ gal+ activation. The clones are further selected by

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cyclohexamide sensitivity and the inserts of the human brain cDNAs are isolated by PCR and sequenced.

Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the following claims.

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CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a nucleotide sequence encoding a protein selected from the group consisting of a normal CalDAG-GEFI protein, a mutant CalDAG-GEFI protein, a normal CalDAG-GEFII protein, and a mutant CalDAG-GEFII protein.
1. 2. An isolated nucleic acid comprising a nucleotide sequence encoding a protein selected from the group consisting of a normal cAMP-GEFI protein, a mutant cAMP-GEFI protein, a normal cAMP-GEFII protein, and a mutant cAMP-GEFII protein.
1. 3. An isolated nucleic acid as in claim 1 wherein said nucleic acid encodes a normal CalDAG-GEF protein and wherein said nucleotide sequence is selected from the group consisting of
  4. (a) a sequence encoding a protein comprising the human CalDAG-GEFI amino acid sequence of SEQ ID NO: 4;
  6. (b) a sequence encoding a protein comprising the murine CalDAG-GEFI amino acid sequence of SEQ ID NO: 2;
  8. (c) a sequence encoding a protein comprising the human CalDAG-GEFII amino acid sequence of SEQ ID NO: 8; and
  10. (d) a sequence encoding a protein comprising the murine CalDAG-GEFII amino acid sequence of SEQ ID NO: 6; and
  12. (e) a sequence encoding a normal CalDAG-GEF protein and capable of hybridizing to a sequence complementary to any sequence of (a) - (d) under stringent hybridization conditions.
1. 4. An isolated nucleic acid as in claim 2 wherein said nucleic acid encodes a normal cAMP-GEF protein and wherein said nucleotide sequence is selected from the group consisting of
  4. (a) a sequence encoding a protein comprising the human cAMP-GEFI amino acid sequence of SEQ ID NO: 12;
  6. (b) a sequence encoding a protein comprising the alternatively spliced human cAMP-GEFI amino acid sequence of SEQ ID NO: 14;
  8. (c) a sequence encoding a protein comprising the rat cAMP-GEFI amino acid sequence of SEQ ID NO: 10;
  10. (d) a sequence encoding a protein comprising the human cAMP-GEFII amino acid sequence of SEQ ID NO: 18;

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12                 (e) a sequence encoding a protein comprising the rat cAMP-GEFII amino acid  
13 sequence of SEQ ID NO: 16; and

14                 (f) a sequence encoding a normal cAMP-GEF protein and capable of hybridizing to a  
15 sequence complementary to any sequence of (a) - (e) under stringent hybridization conditions.

1         5.                 An isolated nucleic acid comprising a nucleotide sequence of at least 8 consecutive  
2 nucleotides selected from the group consisting SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5,  
3 SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO.  
4 17, and a sequence complementary to any of these sequences.

1         6.                 An isolated nucleic acid comprising a nucleotide sequence of at least 10 consecutive  
2 nucleotides selected from the group consisting SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5,  
3 SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO.  
4 17, and a sequence complementary to any of these sequences.

1         7.                 An isolated nucleic acid comprising a nucleotide sequence of at least 15 consecutive  
2 nucleotides selected from the group consisting SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5,  
3 SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO.  
4 17, and a sequence complementary to any of these sequences.

1         8.                 An isolated nucleic acid comprising a nucleotide sequence encoding at least one  
2 functional domain of a CalDAG-GEF protein selected from the group consisting of a normal  
3 CalDAG-GEFI protein, a mutant CalDAG-GEFI protein, a normal CalDAG-GEFII protein, and a  
4 mutant CalDAG-GEFII protein.

1         9.                 An isolated nucleic acid comprising a nucleotide sequence encoding at least one  
2 functional domain of a cAMP-GEF protein selected from the group consisting of a normal  
3 cAMP-GEFI protein, a normal cAMP-GEFII protein, a mutant cAMP-GEFI protein, and a  
4 mutant cAMP-GEFII protein.

1         10.                 An isolated nucleic acid comprising a nucleotide sequence encoding an antigenic  
2 determinant of a CalDAG-GEF protein selected from the group consisting of a normal CalDAG-  
3 GEFI protein, a normal CalDAG-GEFII protein, a mutant CalDAG-GEFI protein, and a mutant  
4 CalDAG-GEFII protein.

1         11.                 An isolated nucleic acid comprising a nucleotide sequence encoding an antigenic  
2 determinant of a cAMP-GEF protein selected from the group consisting of a normal cAMP-GEFI  
3 protein, a normal cAMP-GEFII protein, a mutant cAMP-GEFI protein, and a mutant cAMP-  
4 GEFII protein.

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- 1    12.     A method for identifying an allelic variant or heterospecific homologue of a human  
2    CalDAG-GEF gene comprising:
  - 3       choosing a nucleic acid probe or primer capable of hybridizing to a human CalDAG-
  - 4       GEF gene sequence under stringent hybridization conditions;
  - 5       mixing said probe or primer with a sample of nucleic acids which may contain a  
6       nucleic acid corresponding to said variant or homologue; and
  - 7       detecting hybridization of said probe or primer to said nucleic acid corresponding to  
8       said variant or homologue.
- 1    13.     A method as in claim 12 wherein said human CalDAG-GEF gene sequence is  
2    selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 7.
- 1    14.     A method as in claim 12 wherein said sample comprises a sample of nucleic acids  
2    selected from the group consisting of human genomic DNA, human mRNA, and human cDNA.
- 1    15.     A method as in claim 12 wherein said sample comprises a sample of nucleic acids  
2    selected from the group consisting of mammalian genomic DNA, mammalian mRNA, and  
3    mammalian cDNA.
- 1    16.     A method as in claim 12 wherein said sample comprises a sample of nucleic acids  
2    selected from the group consisting of invertebrate genomic DNA, invertebrate mRNA, and  
3    invertebrate cDNA.
- 1    17.     A method as in claim 12 further comprising the step of isolating said nucleic acid  
2    corresponding to said variant or homologue.
- 1    18.     A method as in claim 12 wherein said nucleic acid is identified by hybridization.
- 1    19.     A method as in claim 12 wherein said nucleic acid is identified by PCR amplification.
- 1    20.     A method for identifying allelic variants or heterospecific homologues of a human  
2    cAMP-GEF gene comprising:
  - 3       choosing a nucleic acid probe or primer capable of hybridizing to a human cAMP-
  - 4       GEF gene sequence under stringent hybridization conditions;
  - 5       mixing said probe or primer with a sample of nucleic acids which may contain a  
6       nucleic acid corresponding to said variant or homologue; and
  - 7       detecting hybridization of said probe or primer to said nucleic acid corresponding to  
8       said variant or homologue.
- 1    21.     A method as in claim 12 wherein said human cAMP-GEF gene sequence is selected  
2    from the group consisting of SEQ ID NO: 11, SEQ ID NO: 13, and SEQ ID NO: 17.

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1 22. A method as in claim 20 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of human genomic DNA, human mRNA, and human cDNA.

1 23. A method as in claim 20 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of mammalian genomic DNA, mammalian mRNA, and  
3 mammalian cDNA.

1 24. A method as in claim 20 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of invertebrate genomic DNA, invertebrate mRNA, and  
3 invertebrate cDNA.

1 25. A method as in claim 20 further comprising the step of isolating said nucleic acid  
2 corresponding to said variant or homologue.

1 26. A method as in claim 20 wherein said nucleic acid is identified by hybridization.

1 27. A method as in claim 20 wherein said nucleic acid is identified by PCR amplification.

1 28. A method for identifying an allelic variant or heterospecific homologue of a human  
2 CalDAG-GEF gene comprising:

3 choosing an antibody capable of selectively binding to a human CalDAG-GEF  
4 protein;

5 mixing said antibody with a sample of proteins which may contain a protein  
6 corresponding to said variant or homologue; and

7 detecting binding of said antibody to said protein corresponding to said variant or  
8 homologue.

1 29. A method as in claim 28 wherein said sample comprises a sample of proteins selected  
2 from the group consisting of human proteins, human fusion proteins, and proteolytic fragments  
3 thereof.

1 30. A method as in claim 28 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of mammalian proteins, mammalian fusion proteins, and  
3 proteolytic fragments thereof.

1 31. A method as in claim 28 wherein said sample comprises a sample of nucleic acids  
2 selected from the group consisting of invertebrate proteins, invertebrate fusion proteins, and  
3 proteolytic fragments thereof.

1 32. A method as in claim 28 further comprising the step of substantially purifying said  
2 protein corresponding to said variant or homologue..

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- 1    33.       A method for identifying an allelic variant or heterospecific homologue of a human  
2    cAMP-GEF gene comprising:
  - 3           choosing an antibody capable of selectively binding to a human cAMP-GEF protein;
  - 4           mixing said antibody with a sample of proteins which may contain a protein
  - 5           corresponding to said variant or homologue; and
  - 6           detecting binding of said antibody to said protein corresponding to said variant or
  - 7           homologue.
- 1    34.       A method as in claim 33 wherein said sample comprises a sample of proteins selected  
2    from the group consisting of human proteins, human fusion proteins, and proteolytic fragments  
3    thereof.
- 1    35.       A method as in claim 33 wherein said sample comprises a sample of proteins selected  
2    from the group consisting of mammalian proteins, mammalian fusion proteins, and proteolytic  
3    fragments thereof.
- 1    36.       A method as in claim 33 wherein said sample comprises a sample of proteins selected  
2    from the group consisting of invertebrate proteins, invertebrate fusion proteins, and proteolytic  
3    fragments thereof.
- 1    37.       A method as in claim 33 further comprising the step of substantially purifying said  
2    protein corresponding to said variant or homologue.
- 1    38.       An isolated nucleic acid comprising an allelic variant or a heterospecific homologue  
2    of a gene selected from the group consisting of a human CalDAG-GEF gene, and a human  
3    cAMP-GEF gene.
- 1    39.       An isolated nucleic acid encoding an allelic variant or heterospecific homologue of a  
2    protein selected from the group consisting of a human CalDAG-GEF protein, and a human  
3    cAMP-GEF protein.
- 1    40.       An isolated nucleic acid comprising a recombinant vector including a nucleotide  
2    sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5,  
3    SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO:  
4    17, and a sequence complementary to any of these sequences.
- 1    41.       An isolated nucleic acid as in claim 40 wherein said vector is an expression vector  
2    and said nucleotide sequence is operably joined to a regulatory region.
- 1    42.       An isolated nucleic acid as in claim 41 wherein said expression vector may express  
2    said nucleotide sequence in mammalian cells.

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- 1    43.       An isolated nucleic acid as in claim 42 wherein said cells are selected from the group  
2       consisting of fibroblast, liver, kidney, spleen, bone marrow, and neurological cells.
- 1    44.       An isolated nucleic acid as in claim 42 wherein said vector is selected from the group  
2       consisting of vaccinia virus, adenovirus, retrovirus, neurotropic viruses, and Herpes simplex.
- 1    45.       An isolated nucleic acid as in claim 41 wherein said expression vector encodes at  
2       least a functional domain of a protein selected from the group consisting of normal CalDAG-  
3       GEFI, a normal CalDAG-GEFII, a mutant CalDAG-GEFI, a mutant CalDAG-GEFII, a normal  
4       cAMP-GEFI, a normal cAMP-GEFII, a mutant cAMP-GEFI, and a mutant cAMP-GEFII.
- 1    46.       An isolated nucleic acid as in claim 41 wherein said vector further comprises  
2       sequences encoding an exogenous protein operably joined to said nucleotide sequence and  
3       whereby said vector encodes a fusion protein.
- 1    47.       An isolated nucleic acid as in claim 46 wherein said exogenous protein is selected  
2       from the group consisting of lacZ, trpE, maltose-binding protein, poly-His tags, and glutathione-  
3       S-transferase.
- 1    48.       An isolated nucleic acid comprising a recombinant expression vector including  
2       nucleotide sequences corresponding to an endogenous regulatory region of a gene selected from  
3       the group consisting of a CalDAG-GEF gene, and a cAMP-GEF gene.
- 1    49.       An isolated nucleic acid as in claim 48 wherein said endogenous regulatory region is  
2       operably joined to a marker gene.
- 1    50.       A host cell transformed with an expression vector of any one of claims 41-49, or a  
2       descendant thereof.
- 1    51.       A host cell as in claim 50 wherein said host cell is selected from the group consisting  
2       of bacterial cells and yeast cells.
- 1    52.       A host cell as in claim 50 wherein said host cell is selected from the group consisting  
2       of fetal cells, embryonic stem cells, zygotes, gametes, and germ line cells.
- 1    53.       A host cell as in claim 50 wherein said cell is selected from the group consisting of  
2       fibroblast, liver, kidney, spleen, bone marrow and neurological cells.
- 1    54.       A host cell as in claim 50 wherein said cell is an invertebrate cell.
- 1    55.       A non-human animal model for cancer, wherein a genome of said animal, or an  
2       ancestor thereof, has been modified by at least one recombinant construct, and wherein said  
3       recombinant construct has introduced a modification selected from the group consisting of

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- 4                             (a) insertion of nucleotide sequences encoding at least a functional domain of  
5                             a heterospecific normal CalDAG-GEF gene;  
6                             (b) insertion of nucleotide sequences encoding at least a functional domain of  
7                             a heterospecific mutant CalDAG-GEF gene;  
8                             (c) insertion of nucleotide sequences encoding at least a functional domain of  
9                             a conspecific homologue of a heterospecific mutant CalDAG-GEF gene;  
10                           (d) inactivation of an endogenous CalDAG-GEF gene;  
11                           (e) insertion of nucleotide sequences encoding at least a functional domain of  
12                             a heterospecific normal cAMP-GEF gene;  
13                           (f) insertion of nucleotide sequences encoding at least a functional domain of a  
14                             heterospecific mutant cAMP-GEF gene;  
15                           (g) insertion of nucleotide sequences encoding at least a functional domain of  
16                             a conspecific homologue of a heterospecific mutant cAMP-GEF gene; and  
17                           (h) inactivation of an endogenous cAMP-GEF gene.

1       56.     A non-human animal model as in claim 55 wherein said cancer is related to the Ras-pathway.

1       57.     A non human animal model as in claim 56 wherein said cancer is selected from the  
2                             group consisting of lung cancer, pancreatic cancer, breast cancer, colorectal cancer, and myeloid  
3                             leukemia.

1       58.     An animal model as in claim 55 wherein said modification is an insertion of a  
2                             nucleotide sequence encoding at least a functional domain of a protein selected from the group  
3                             consisting of a normal human CalDAG-GEF, and a normal cAMP-GEF gene.

1       59.     An animal model as in claim 55 wherein said modification is an insertion of a  
2                             nucleotide sequence encoding at least a functional domain of a protein selected from the group  
3                             consisting of a mutant human CalDAG-GEF, and a mutant human cAMP-GEF gene.

1       60.     An animal as in claim 55 wherein said animal is selected from the group consisting of  
2                             rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates.

1       61.     An animal as in claim 55 wherein said animal is an invertebrate.

1       62.     A method for producing at least a functional domain of a protein selected from the  
2                             group consisting of a CalDAG-GEF protein, and a cAMP-GEF protein, said method comprising  
3                             culturing a host cell of any of claims 50-54 under suitable conditions to produce said protein by  
4                             expressing said nucleic acid.

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1 63. A substantially pure preparation of a protein selected from the group consisting of a  
2 normal CalDAG-GEF protein, a mutant CalDAG-GEF protein, a normal cAMP-GEF protein,  
3 and a mutant cAMP-GEF protein.

1 64. A substantially pure preparation as in claim 63 wherein said protein comprises a  
2 normal protein selected from the group consisting of

- 3 (a) a protein comprising the amino acid sequence of SEQ ID NO: 2;
- 4 (b) a protein comprising the amino acid sequence of SEQ ID NO: 4;
- 5 (c) a protein comprising the amino acid sequence of SEQ ID NO: 6;
- 6 (d) a protein comprising the amino acid sequence of SEQ ID NO: 8;
- 7 (e) a protein comprising the amino acid sequence of SEQ ID NO: 10;
- 8 (f) a protein comprising the amino acid sequence of SEQ ID NO: 12;
- 9 (g) a protein comprising the amino acid sequence of SEQ ID NO: 14;
- 10 (h) a protein comprising the amino acid sequence of SEQ ID NO: 16; and
- 11 (i) a protein comprising the amino acid sequence of SEQ ID NO: 18.

1 65. A substantially pure preparation of a polypeptide comprising an amino acid sequence  
2 of at least 4 consecutive amino acid residues selected from the group consisting of SEQ ID NO:  
3 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID  
4 NO: 14, SEQ ID NO: 16, and SEQ ID NO: 18.

1 66. A substantially pure preparation of a polypeptide comprising an amino acid sequence  
2 of at least 10 consecutive amino acid residues selected from the group consisting of SEQ ID NO:  
3 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID  
4 NO: 14, SEQ ID NO: 16, and SEQ ID NO: 18.

1 67. A substantially pure preparation of a polypeptide comprising an amino acid sequence  
2 of at least 15 consecutive amino acid residues selected from the group consisting of SEQ ID NO:  
3 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID  
4 NO: 14, SEQ ID NO: 16, and SEQ ID NO: 18.

1 68. A substantially pure preparation of a polypeptide comprising at least one functional  
2 domain of a protein selected from the group consisting of a normal CalDAG-GEF protein, a  
3 mutant CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF protein.

1 69. A substantially pure preparation of a polypeptide comprising an antigenic determinant  
2 of a protein selected from the group consisting of a normal CalDAG-GEF protein, a mutant  
3 CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF protein.

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1 70. A method of producing antibodies which selectively bind to a CalDAG-GEF protein  
2 comprising the steps of

3 administering an immunogenically effective amount of a CalDAG-GEF immunogen  
4 to an animal;

5 allowing said animal to produce antibodies to said immunogen; and  
6 obtaining said antibodies from said animal or from a cell culture derived therefrom.

1 71. A method of producing antibodies which selectively bind to a cAMP-GEF protein  
2 comprising the steps of

3 administering an immunogenically effective amount of a cAMP-GEF immunogen to  
4 an animal;

5 allowing said animal to produce antibodies to said immunogen; and  
6 obtaining said antibodies from said animal or from a cell culture derived therefrom.

1 72. A substantially pure preparation of an antibody which selectively binds to an  
2 antigenic determinant of a protein selected from the group consisting of a normal CalDAG-GEF  
3 protein, a mutant CalDAG-GEF protein, a normal cAMP-GEF protein, and a mutant cAMP-GEF  
4 protein.

1 73. A substantially pure preparation of an antibody as in claim 72 wherein said antibody  
2 selectively binds to an antigenic determinant of a mutant CalDAG-GEF and fails to bind to a  
3 normal CalDAG-GEF protein.

1 74. A substantially pure preparation of an antibody as in claim 72 wherein said antibody  
2 selectively binds to an antigenic determinant of a mutant cAMP-GEF and fails to bind to a  
3 normal cAMP-GEF protein.

1 75. A cell line producing an antibody of any one of claims 72-74.

1 76. A method for identifying compounds which can modulate the expression of a  
2 CalDAG-GEF gene comprising:

3 contacting a cell with a test candidate wherein said cell includes a regulatory region of  
4 a CalDAG-GEF gene operably joined to a coding region; and  
5 detecting a change in expression of said coding region.

1 77. A method for identifying compounds which can modulate the expression of a cAMP-  
2 GEF gene comprising:

3 contacting a cell with a test candidate wherein said cell includes a regulatory region of  
4 a cAMP-GEF gene operably joined to a coding region; and

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5           detecting a change in expression of said coding region.

1   78.       A method as in claim 76 or 77 wherein said change comprises a change in a level of  
2   an mRNA transcript encoded by said coding region.

1   79.       A method as in claim 78 wherein said change comprises a change in a level of a  
2   protein encoded by said coding region.

1   80.       A method as in claim 78 wherein said change is a result of an activity of a protein  
2   encoded by said coding region.

1   81.       A method as in claim 78 wherein said coding region encodes a marker protein  
2   selected from the group consisting of  $\beta$ -galactosidase, alkaline phosphatase, green fluorescent  
3   protein, and luciferase.

1   82.       A method for identifying compounds which can selectively bind to a CalDAG-GEF  
2   protein comprising the steps of

3           providing a preparation including at least one CalDAG-GEF component;  
4           contacting said preparation with a sample including at least one candidate compound;

5           and

6           detecting binding of said CalDAG-GEF component to said candidate compound.

1   83.       A method for identifying compounds which can selectively bind to a cAMP-GEF  
2   protein comprising the steps of

3           providing a preparation including at least one cAMP-GEF component;  
4           contacting said preparation with a sample including at least one candidate compound;

5           and

6           detecting binding of said cAMP-GEF component to said candidate compound.

1   84.       The method in claim 82 wherein said binding to said CalDAG-GEF component is  
2   detected by an assay selected from the group consisting of: affinity chromatography, co-  
3   immunoprecipitation, a Biomolecular Interaction Assay, and a yeast two-hybrid system.

1   85.       The method in claim 83 wherein said binding to said cAMP-GEF component is  
2   detected by an assay selected from the group consisting of: affinity chromatography, co-  
3   immunoprecipitation, a Biomolecular Interaction Assay, and a yeast two-hybrid system.

1   86.       A method of identifying compounds which can modulate activity of a CalDAG-GEF  
2   comprising the steps of

3           providing a cell expressing a normal or mutant CalDAG-GEF gene;  
4           contacting said cell with at least one candidate compound; and

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- 5           detecting a change in a marker of said activity.
- 1   87.       A method of identifying compounds which can modulate activity of a cAMP-GEF  
2   comprising the steps of  
3       providing a cell expressing a normal or mutant cAMP-GEF gene;  
4       contacting said cell with at least one candidate compound; and  
5       detecting a change in a marker of said activity.
- 1   88.       A method as in claim 86 wherein measurement of said marker indicates a difference  
2   between cells bearing an expressed mutant CalDAG-GEF gene and otherwise identical cells free  
3   of an expressed mutant CalDAG-GEF gene.
- 1   89.       A method as in claim 86 wherein said change comprises a change in a non-specific  
2   marker of cell physiology selected from the group consisting of pH, intracellular calcium, cyclic  
3   AMP levels, GTP/GDP ratios, phosphatidylinositol activity, and protein phosphorylation.
- 1   90.       A method as in claim 86 wherein said change comprises a change in expression of  
2   said CalDAG-GEF.
- 1   91.       A method as in claim 86 wherein said change comprises a change in occurrence or  
2   rate of apoptosis or cell death.
- 1   92.       A method as in claim 86 wherein said cell is a cell cultured *in vitro*.
- 1   93.       A method as in claim 92 wherein said cell is a transformed host cell of any one of  
2   claims 50-54.
- 1   94.       A method as in claim 92 wherein said cell is explanted from a host bearing at least  
2   one mutant CalDAG-GEF gene.
- 1   95.       A method as in claim 92 wherein said cell is explanted from a transgenic animal of  
2   any one of claims 55-61.
- 1   96.       A method as in claim 86 wherein said cell is a cell in a live animal.
- 1   97.       A method as in claim 96 wherein said cell is a cell of a transgenic animal of any one  
2   of claims 55-61.
- 1   98.       A method as in claim 86 wherein said cell is in a human subject in a clinical trial.
- 1   99.       A method as in claim 87 wherein measurement of said marker indicates a difference  
2   between cells bearing an expressed mutant cAMP-GEF gene and otherwise identical cells free of  
3   an expressed mutant cAMP-GEF gene.

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- 1    100.       A method as in claim 87 wherein said change comprises a change in a non-specific  
2    marker of cell physiology selected from the group consisting of pH, intracellular calcium, cyclic  
3    AMP levels, GTP/GDP ratios, phosphatidylinositol activity, and protein phosphorylation.
- 1    101.       A method as in claim 87 wherein said change comprises a change in expression of  
2    said cAMP-GEF.
- 1    102.       A method as in claim 87 wherein said change comprises a change in occurrence or  
2    rate of apoptosis or cell death.
- 1    103.       A method as in claim 87 wherein said cell is a cell cultured *in vitro*.
- 1    104.       A method as in claim 103 wherein said cell is a transformed host cell of any one of  
2    claims 50-54.
- 1    105.       A method as in claim 103 wherein said cell is explanted from a host bearing at least  
2    one mutant cAMP-GEF gene.
- 1    106.       A method as in claim 103 wherein said cell is explanted from a transgenic animal of  
2    any one of claims 55-61.
- 1    107.       A method as in claim 87 wherein said cell is a cell in a live animal.
- 1    108.       A method as in claim 107 wherein said cell is a cell of a transgenic animal of any one  
2    of claims 55-61.
- 1    109.       A method as in claim 87 wherein said cell is in a human subject in a clinical trial.
- 1    110.       A diagnostic method for determining if a subject bears a mutant CalDAG-GEF gene  
2    comprising the steps of
  - 3           providing a biological sample of said subject; and
  - 4           detecting in said sample a mutant CalDAG-GEF nucleic acid, a mutant CalDAG-GEF  
5           protein, or a mutant CalDAG-GEF activity.
- 1    111.       A method as in claim 111, wherein a mutant CalDAG-GEF nucleic acid is detected  
2    by an assay selected from the group consisting of direct nucleotide sequencing, probe specific  
3    hybridization, restriction enzyme digest and mapping, PCR mapping, ligase-mediated PCR  
4    detection, RNase protection, electrophoretic mobility shift detection, and chemical mismatch  
5    cleavage.
- 1    112.       A method as in claim 110, wherein a mutant CalDAG-GEF protein is detected by an  
2    assay selected from the group consisting of an immunoassay, a protease assay, and an  
3    electrophoretic mobility assay.

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1 113. A diagnostic method for determining if a subject bears a mutant cAMP-GEF gene  
2 comprising the steps of

3 providing a biological sample of said subject; and  
4 detecting in said sample a mutant cAMP-GEF nucleic acid, a mutant cAMP-GEF  
5 protein, or a mutant cAMP-GEF activity.

1 114. A method as in claim 113, wherein a mutant cAMP-GEF nucleic acid is detected by  
2 an assay selected from the group consisting of direct nucleotide sequencing, probe specific  
3 hybridization, restriction enzyme digest and mapping, PCR mapping, ligase-mediated PCR  
4 detection, RNase protection, electrophoretic mobility shift detection, and chemical mismatch  
5 cleavage.

1 115. A method as in claim 113, wherein a mutant cAMP-GEF protein is detected by an  
2 assay selected from the group consisting of an immunoassay, a protease assay, and an  
3 electrophoretic mobility assay.

1 116. A pharmaceutical preparation comprising a substantially pure CalDAG-GEF protein  
2 and a pharmaceutically acceptable carrier.

1 117. A pharmaceutical preparation comprising a substantially pure cAMP-GEF protein and  
2 a pharmaceutically acceptable carrier.

1 118. A pharmaceutical preparation comprising an expression vector operably encoding a  
2 protein selected from the group consisting of a CalDAG-GEF protein, and a cAMP-GEF protein,  
3 wherein said expression vector may express said CalDAG-GEF protein or said cAMP-GEF  
4 protein in a human subject, and a pharmaceutically acceptable carrier.

1 119. A pharmaceutical preparation comprising an expression vector operably encoding a  
2 CalDAG-GEF antisense sequence, wherein said expression vector may express said CalDAG-  
3 GEF antisense sequence in a human subject, and a pharmaceutically acceptable carrier.

1 120. A pharmaceutical preparation comprising an expression vector operably encoding a  
2 cAMP-GEF antisense sequence, wherein said expression vector may express said cAMP-GEF  
3 antisense sequence in a human subject, and a pharmaceutically acceptable carrier.

1 121. A pharmaceutical preparation comprising a substantially pure antibody, and a  
2 pharmaceutically acceptable carrier,

3 wherein said antibody selectively binds to a mutant protein selected from the group  
4 consisting of a mutant CalDAG-GEF protein, and a mutant cAMP-GEF protein.

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- 1 122. A pharmaceutical preparation as in claim 121 wherein said preparation is essentially  
2 free of an antibody which selectively binds a normal CalDAG-GEF protein.
- 1 123. A pharmaceutical preparation as in claim 121 wherein said preparation is essentially  
2 free of an antibody which selectively binds a normal cAMP-GEF protein.
- 1 124. A pharmaceutical preparation comprising a substantially pure preparation of an  
2 antigenic determinant of a mutant CalDAG-GEF protein or a mutant cAMP-GEF protein.
- 1 125. A pharmaceutical preparation as in claim 124 wherein said preparation is essentially  
2 free of an antigenic determinant of a normal CalDAG-GEF protein.
- 1 126. A pharmaceutical preparation as in claim 124 wherein said preparation is essentially  
2 free of an antigenic determinant of a normal cAMP-GEF protein.
- 1 127. A method of treatment for a patient bearing a mutant CalDAG-GEF gene comprising  
2 the step of administering to said patient a therapeutically effective amount of the pharmaceutical  
3 preparation of claim 116.
- 1 128. A method for identifying compounds according to claim 82, wherein the CalDAG-  
2 GEF component is a CalDAG-GEF domain selected from the group consisting of SCR1, SCR2,  
3 SCR3, DAG-binding and an EF hand domain.
- 1 129. A substantially pure preparation of a polypeptide comprising a domain selected from  
2 the group consisting of a CalDAG-GEF SCR1 domain, a CalDAG-GEF SCR2 domain,  
3 CalDAG-GEF SCR3 domain, CalDAG-GEF DAG-binding domain, CalDAG-GEF EF hand  
4 domain.
- 1 130. A substantially pure preparation of a polypeptide comprising a domain selected from  
2 the group consisting of a cAMP-GEF SCR1 domain, a cAMP-GEF SCR2 domain, cAMP-GEF  
3 SCR3 domain, cAMP-GEF cAMP-binding domain.

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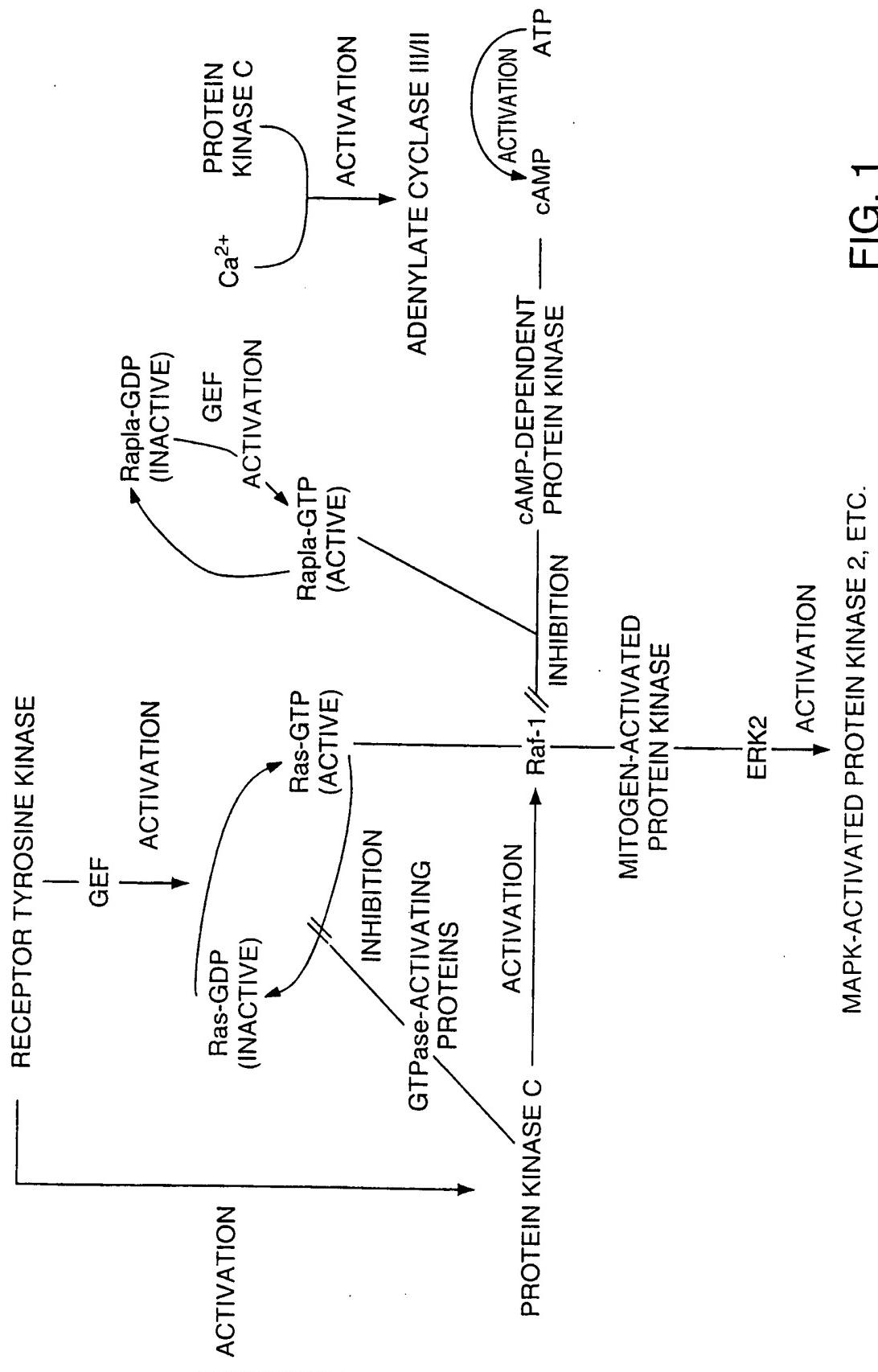


FIG. 1

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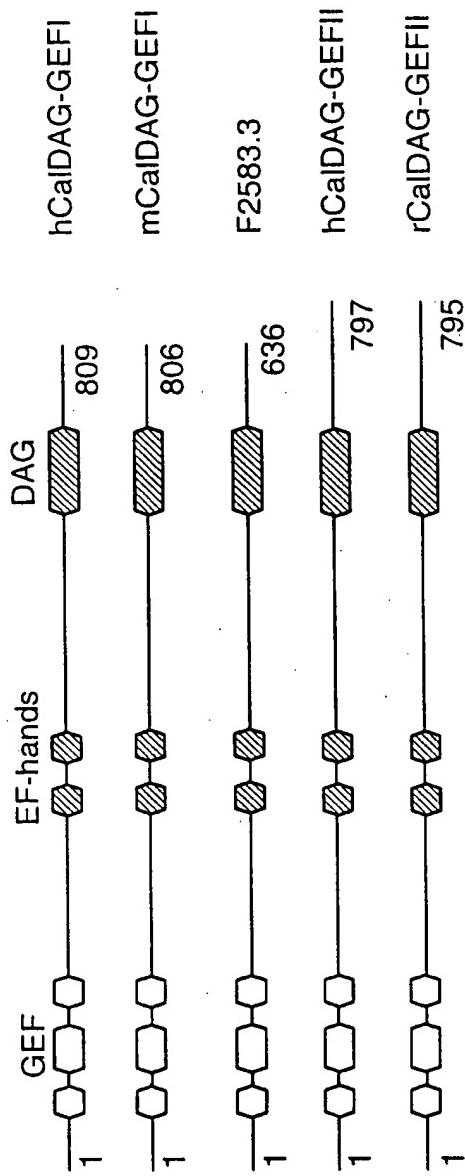


FIG. 2A

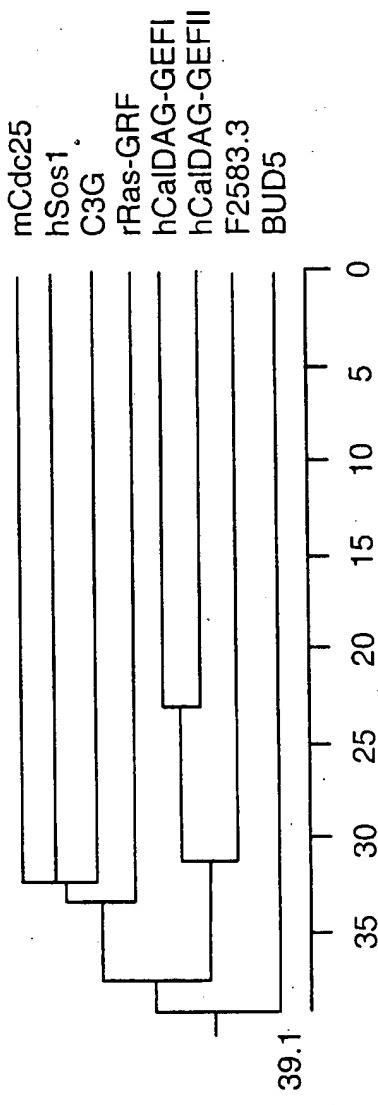


FIG. 2B

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FIG. 2D-1	FIG. 2D-2
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FIG. 2D

hCalDAG-GEFI	M A G T L D K G C T V E E L L R G C I E A F D D S G K V R D P Q L V R M F L M M H P W Y I P S S
mcalDAG-GEFI	M A S T L D K G C T V E E L L R G C I E A F D D S G K V R D P Q L V R M F L M M H P W Y I P S S
hCalDAG-GEFI	K E L K A L L D Q E G N R R H S S S L I D I D S V P T Y K W K R Q V T Q R N P V G Q K K R K M S L L F
mcalDAG-GEFI	K E L K A L L D Q E G N R R H S S S L I D I E S V P T Y K W K R Q V T Q R N P V E Q K K R K M S L L F
hCalDAG-GEFI	N S V S Q W V Q L M I L S K P T A P Q R A L V I T H F V H V A E K L L Q L Q N F N T L M A V V G G L
mcalDAG-GEFI	N S V S Q W V Q L M I L S K P T A T Q R A L V I T H F V H V A E K L L Q L Q N F N T L M A V V G G L
hCalDAG-GEFI	F P I L G V H L K D L V A L Q L A L P D W L D P A R T R I L N G A K M K Q L F S I L E E L A M V T S L
mcalDAG-GEFI	F P I L G V H L K D L V A L Q L A L P D W L D P G R T R I L N G A K M R Q L F S I L E E L A M V T S L
hCalDAG-GEFI	P P R P P V L E E W Y S A A K P K L D Q A L V V E H I E K M V E S V E R N F D V D G D G H I S Q E E
mcalDAG-GEFI	P P R P P V L E E W Y S Y A K P K L D Q A L V A E H I E K M V E S V F R N F D V D G D G H I S Q E E
hCalDAG-GEFI	F Q E S N S L R P V A C R H C K A L I L G I Y K Q G L K C R A C G V N C H K Q C K D R L S L E C R R
mcalDAG-GEFI	F Q E S N S L R P V A C R H C K A L I L G I Y K Q G L K C R A C G V N C H K Q C K E R L S L E C R R
hCalDAG-GEFI	E D G V F D I H I
mcalDAG-GEFI	E D G V F D I H I

FIG. 2D-1

QLAAKLLH <sup>I</sup> YQQSRKDNNSSN <sup>S</sup> LQYKTC <sup>H</sup> LVR <sup>I</sup> WISAFPAEFDLNPELAEQI	100
QLA[S]KLLH[ <sup>E</sup> ]YQQSRKDNNSSN <sup>S</sup> LQYKTC <sup>H</sup> LVR <sup>I</sup> W[ <sup>V</sup> ]SAFPAAFDLNPELAE[ <sup>P</sup> I]	100
DHLEPME <sup>A</sup> HLTYLEYRSFCKILFQDYHSFVTHGCC <sup>T</sup> VDNPVLERFISLF	200
DHLEPME <sup>A</sup> HLTYLEYRSFCKILFQDYHSFVTHGCC <sup>T</sup> VDNPVLERFISLF	200
SHSSISRLKETHSHVSPE <sup>T</sup> IKLWEGLTELVTATGNYGNYR <sup>R</sup> RLAACVGFR	300
SHSSISRLKETHSHVS <sup>P</sup> D <sup>T</sup> IKLWEGLTELVTATGNY[ <sup>S</sup> ]NYRRRLAACVGFR	300
RPPVQANPDLLSLLT <sup>V</sup> SLDQYQTEDELYQLSSLQREP <sup>R</sup> SKSSPTSP <sup>T</sup> C <sup>T</sup> P	400
RPPVQAKPSLLSLLT <sup>V</sup> SLDQYQTEDELYQLSSLQREP <sup>R</sup> SKSSPTSP <sup>T</sup> C <sup>T</sup> P	400
FQIIRGNFPYILSAFGDLOQ <sup>N</sup> QDGCI <sup>S</sup> REEMVSYFLRSSSVLGGRMGFVHN	500
FQIIRGNFPYILSAFGDLOQ <sup>N</sup> QDGCI <sup>S</sup> REEM[ <sup>M</sup> ]SYFLRSSSVLGGRMGFVHN	500
RAQS <sup>V</sup> SLLEGSSAPSPSPMHSHHRAFSFSSLPRPGRRGSRP <sup>P</sup> EIREEEVQT <sup>V</sup>	600
RAQS <sup>V</sup> SLLEGSSAPSPSP[ <sup>T</sup> H] <sup>H</sup> [ <sup>G</sup> ]SRRP <sup>P</sup> GRRG <sup>S</sup> RP <sup>P</sup> EIREEEVQ[S]V	599
	609
	608

FIG. 2D-2

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	20	30	
F2583.3	A V F K H Y D H D R D G F I S Q E E F Q	473	
hCalDAG-GEFI	S V F R N F D V D G D G H I S Q E E F Q	452	
hCalDAG-GEFII	S V F K N Y D H D Q D G Y I S Q E E F E	447	
hCalmodulin	E A F S L F D K D G D G T I T K F I L G	34	
hCalbindin D28K	L M L K L F D S N N D G K L E L T E M A	168	
hCalcineurin B	F A F R I Y D M D K D G Y I S N G E I F	113	
hParvalbumin $\alpha$	K V F H M I D K D R S G F I E D E I L G	65	
hTroponin C	E C F R I F D R K A D G Y I D P E E I L A	117	

FIG. 2E

	10	20	30	40	50
F2583.3	H N F H E T T F I L T P T T C N H C N K L L W G I L R Q G F K C K D C G L A V H S C C K S N A V A E C	570			
hCalDAG-GEFI	H N F Q E S N S L R P V A C R H C K A L I I L G I Y K Q G E L K C R A C G V N C H K Q C K D R L S V E C	548			
hCalDAG-GEFII	H N F Q E T T Y L K P T F C D N C A G F L W G V I K Q G Y R C C D C C M N C H K Q C K D L V V F E C	542			
hPKC $\alpha$	H K F I A R F F K Q P T F C S H C T D F I W G F G K Q G F Q C Q V C C F V V H K R C H E F V T F S C	86			
hPKC $\beta$ 1	H K F I A R F F K Q P T F C S H C T D F I W G I G K Q G I Q C Q V C C F V V H K R C H E F V T F S C	86			
hPKC $\gamma$	H K F I A R F F K Q P T F C S H C T D F I W G I G K Q G I Q C Q V C S E V V H R C H E F V T F E C	85			

FIG. 2F

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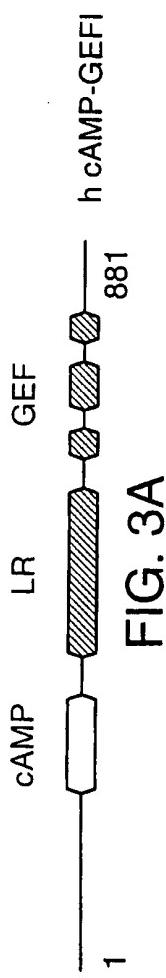


FIG. 3A

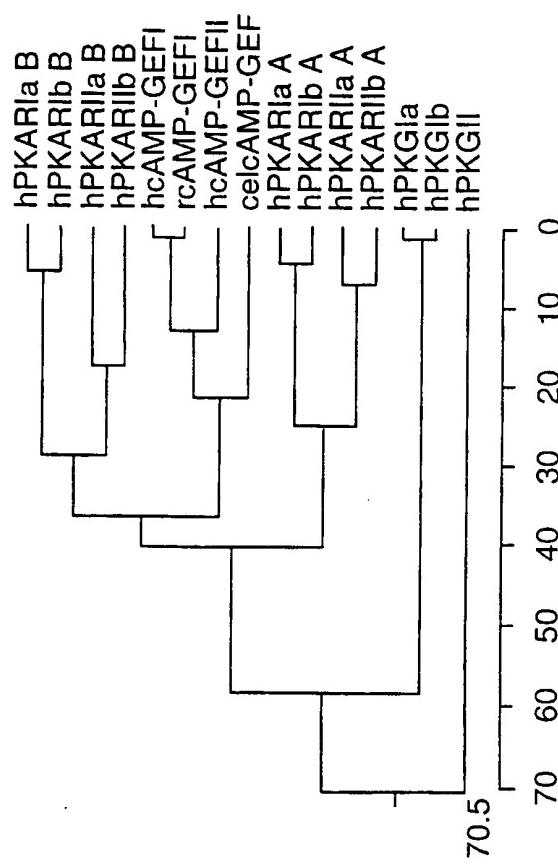


FIG. 3B

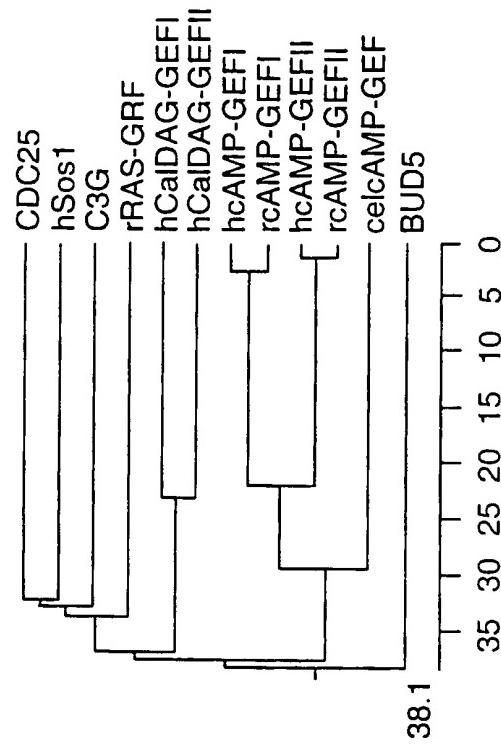


FIG. 3C

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SCR 1	10	20	30	40
hcAMP-GEFI	I D I V S A K D I A G Q L T D R W S L F N S I	639	P V I P F M P L L K D M T F I H E G N K T	789
rcAMP-GEFI	I D I V S A K D I A G Q L T D R D W S L F N R I	642	P V I P F M P L L K D M T F I H E G N K T	792
hcAMP-GEFII	F E L M S S K D I D S Q E L I D P M E L S E R E F T Y L R R S E C R I	1010	P L I P F M P L L I K D M T F I H E G N K T	940
rcAMP-GEFII	F E L M S S K D I D S Q E L I D P M E L S E R E F T Y L R R S E C R I	173	P L I P F M P L L I K D M T F I H E G N K T	365
celcAMP-GEF	I H L I D F D M L E P M E L S E R E F T Y L R R S E C R I	224	F R E P I L G V H L K D L I S L Y E A M P D	1159
hCalDAG-GEFI	F D D N L E P M E L S E R E F T Y L R R S E C R I	224	F R E P I L G V H L K D L I S L Y E A M P D	320
hCalDAG-GEFII	F D D N L E P M E L S E R E F T Y L R R S E C R I	173	F R E P I L G V H L K D L I S L Y E A M P D	371
C3G	I N D F N S N E I A T Q I T V I S S D L Y L R I	859	P C I P Y L G V Y I S D L T F T F V G N P D	1001
CDC25	I L D I D P Y T T I A T Q I T V I S S D L Y L R I	1323	A C V P F F E G V Y I S D L T F T F V G N P D	1474
rRas-GRF	F E N N S A M E I A T Q I T V I S S D L Y L R I	1028	P C V P F F G S Y L T N I L K T E E G N P E	1177
hSos1	I L T L N P I E I A T Q I T V I S S D L Y L R I	799	P C V P F F G S Y L T N I L K T E E G N P E	946
BUD5	A L N V S P W S I A K T L L I S S D L Y L R I	327	P C V P F F G S Y L T N I L K T E E G N P E	644

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SCR 2	10	20	30	40
hcAMP-GEFI	R A Q L L K K F I K L R K F I K L A A H L K E Q K N I N S F F A V M	731	G L S N S P I S R L A N T W	734
rcAMP-GEFI	R V Q L L K K F I K L R K F I K L A A H C K E Y K N I N S F F A I V M	734	G L S N S A I V S R L A L T W	882
hcAMP-GEFII	R V Q L L K K F I K L R K F I K L A A H C K E Y K N I N S F F G I V M	882	G L S N V A E S S I S R L A L T W	307
rcAMP-GEFII	R M E I L K K F I K I S I A T I A R E Y R D I L I T V F A I V M	307	G L S N V A E S S I S R L A L T W	1101
celcAMP-GEF	R A I V I T H F V H V A Q K L N O Q L O N F N E S Y L A I T S A I D S A P I R E -	262	G L S N V A E S S I S R L A L T W	262
hCalDAG-GEFI	R A E V F I K F I Q V A Q K L N O Q L O N F N E S Y L A I T S A I D S A P I R E -	313	G L S N V A E S S I S R L A L T W	313
hCalDAG-GEFII	R E R I I K F I K I M K H K K L N O Q L O N F N E S Y L A I T S A I D S A P I R E -	946	G L S N V A E S S I S R L A L T W	946
C3G	R S K E L T Q Y E V T V A Q H C K E L M N Y N A V L E I T S S I N R S A I E R I L K K T W	1415	G L S N V A E S S I S R L A L T W	1415
CDC25	R A S T K W V A V A D I C R C L M N Y N A V L E V V S A M N S S P V Y R L D K T F	1119	G L S N V A E S S I S R L A L T W	1119
rRas-GRF	R V A V V S R I I E I L Q V F Q E I L Q V A L A C L Y L R N I N S L A S S I T S E Q N H S I R L S L P I	890	G L S N V A E S S I S R L A L T W	890
hSos1	Q T N T I S Y Y W I Q V A L A C L Y L R N I N S L A S S I T S E Q N H S I R L S L P I	406	G L S N V A E S S I S R L A L T W	406
BUD5				

FIG. 3D

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	A									
hcAMP-GEF I	L	F	S	Q	G	D	K	T	S	W
hcAMP-GEF II	I	F	N	Q	G	D	K	T	S	W
hcAMP-GEF II	I	F	N	Q	G	E	I	G	V	Y
celcAMP-GEF	V	F	R	Q	G	G	E	I	G	V
hPKARIIa A	V	I	D	Q	G	D	E	G	P	G
hPKARIIa A	V	I	D	Q	G	D	E	F	F	D
hPKARIIa B	I	V	V	I	V	D	N	F	Y	V
hPKARIIa B	I	I	T	Q	E	G	Q	A	D	S
hPKGIIa	I	I	K	E	E	G	D	V	G	S
hPKGIIb	I	I	K	Q	E	G	D	V	G	S
hPKGII	I	I	K	E	E	G	D	V	G	S

RE YNC H F L R V D K Q D F	300
RE YNC H F L R V D K Q D F	300
RE YNC H F L R V D K E D F	453
RE YNC H F L R V D K H H F	667
RE YNC H F L R V D S Y 231	
RE YNC H F L R V D R V T F	237
RE YNC H F L R V D R P R F	355
RE YNC H F L R V D V Q A F	367
RE YNC H F L R V D R Q C F	197
RE YNC H F L R V D R Q C F	212
RE YNC H F L R V D R K V F	262

A

FIG. 3F-1	FIG. 3F-2	FIG. 3F-3
-----------	-----------	-----------

FIG. 3F

Best Available Copy

FIG. 3E

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hcAMP-GEFI	M V L - - - - -
hcAMP-GEFII	M V A A H A A H S S S S S A E W I A C L D K
hcAMP-GEFI	- - - - - - - - - H Q H P
hcAMP-GEFII	Y A V L A G S L D V K V S E T S S H Q D A
hcAMP-GEFI	- - - - - - - - - T P
hcAMP-GEFII	P Y G V M E T G S M M D R I P D K K N T P
hcAMP-GEFI	L V D   G I L A L G L G   V H S R   S Q   Q V V G   I
hcAMP-GEFII	L V D W M M Q Q T P C V H S R T Q A V G M
hcAMP-GEFI	S Q   R G P D A   L L T V A   L R K P P G Q R T
hcAMP-GEFII	S Q M G P D A M M R W I L R K P P G Q R T
hcAMP-GEFI	G S N K V V   T N G K G L V   T T L H K G D D
hcAMP-GEFII	G S N S V V   I Y G K G V V   C T L H K G D D
hcAMP-GEFI	A S Q   G A - G P S R P P T P G R N R Y T V
hcAMP-GEFII	V P A G   N R R S N Q G N S Q P Q Q K Y T V
hcAMP-GEFI	G G   S E Q E R S T Y V C M E R Q Q I L   R L
hcAMP-GEFII	Q G   T E Q E K K D Y A L M N K K K V I R L
hcAMP-GEFI	S P Q   K K A R M L   P V W L P N Q   D K   P L P
hcAMP-GEFII	A P Q   R K H K V L   L Q Q F N T G D K R - A
hcAMP-GEFI	G   D A I G   L Q   P D A R G   V A T S   L G L N E
hcAMP-GEFII	G   K K V V   L K   P N D V S   V F T F L T I N G
hcAMP-GEFI	H Y V L   G P Q H L R D V   T T A K L E R F   N
hcAMP-GEFII	Y M T F   Q R M N F K K -   T T A K L D L F   L
hcAMP-GEFI	R L A M   T W E R   L P H K   K V R   K L Y   S A L K
hcAMP-GEFII	R L A L   T W E K   L P S K   F K K F Y A E F K
hcAMP-GEFI	A   A R M   L H H C   R S   R N P V   P L S   F   L R S
hcAMP-GEFII	T   A R T   V R Y Y   R S Q - - -   P F N   F - - -

FIG. 3F-1

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R R M H R P R S - - - - -  
 R F L E K S S E D V D I I F T R L K E V K A F E K F H P N L L M  
 S - - C I Q G - - - - -  
 V T I C T L G I G T A F G E S I L D N T F R N A T I V T R E S S  
 L T N S E E S L D F S E S L E Q A S T E R V L R A G R Q L K Q N I  
 L I E P H V P L R P A N T I T K V P S E K T L R A G K I L R N A I  
 C Q V L L D K G A L C H V K K D W A F Q D K D A Q F Y R F P G P E  
 W Q V L L K D G V L N H V D Q E H N F Q D K - Y L F Y R F L D D E  
 D E E L D L I F E E L L H I K A V A N L S N S V K R E L A A V L L  
 V D D L E I I Y E E L L H I K A L S N L S T T V R R E L A G V L I  
 F G Q L A L V N D A P R A A T I I L R E Y N C H F L R V D K Q D F  
 F G K L A L V N D A P R A A S I V L R E D N C H F L R V D K E D F  
 M S G T P D K I L E L L L E A N G L D S E A H D P K E T T L S D F  
 M S G T P E K I L E H F L E T I R L E A T L N E A T D S V L N D F  
 V S Q W V A L Y G S M L H T D P V A T S F L M K L S D L V G R D T  
 V L Q W A A M Y G D L L Q E D D V S K A F L E E F Y V S V S D D A  
 G S S C A I Q V G D K V P Y D I C R P D H S V L T L Q L P V T A S  
 Q K R Q P I R S S D E V L F K V Y C K D H T T T T R V P V A T S  
 R L F V V N P Q E V H E L I P H P D Q L G P T V G S A E G L D L V  
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 R R F N E L O Q Y W V A T E L C L C P V P G P R A Q L L K K F I K L  
 R R F N E I Q F W V V T E I C I C L C S Q L B K R V Q L L K K F I K I  
 R L L D P S W N H R V Y R L A L A K L S P P Y I P F M P L L L K D  
 S L M D P S R N H R A Y R L T V A K L E P P L I P F M P L L I K D  
 R V S H L K G D S Q V A R I S T C S E Q S L S T K S P A S T W A Y  
 - - - - - D A A Q A N K N H Q D V R S Y

**FIG. 3F-2**

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- - - C S Y Q L L L K - - - - - - - - -	20
I C L C G Y Y E N L K K G I T L F R Q G D I G T N W	80
- L R - - - - - W - - - - - - - - -	32
L L R I E Q K D F K A L W E K Y R Q Y M A G L L A P	160
L A T C P N L I R D R K Y H L R L Y R Q C C S G R E	92
L S R A P H M I R D R K Y H L K T Y R Q C C V G T E	240
F E - - P V G T H E M - - - E E E L A E A A V A L L	166
H E D A P L P T E E E K K E C D E E L Q D T M L L	319
F E P H S K A G T V L F S Q G D K G T S W Y X X W K	246
F E S H A K G G T V L F N Q G E E G T S W Y X X L K	399
N R I I K D V E A K T M K L E G K G K V V L V L K R	326
N R I L R D V E A N T V K L K G K D Q D V L V L K K	479
L L T H R V F M P S A Q L C A A L L H H F H V E P A	405
I M M H C V F M P N T Q L C P A L V A H Y H A Q P S	559
R L S N L L R E Q W P E R R R C H R L E N G C G M A	485
R M I A A L K E Q L P E L E K I V K Q I S E - D A K	638
V R E V M A A L A Q E D G W T K G Q V L V K V N S A	565
V K E V I S A V A D K L G S G E G L I I V K M S S G	717
S A K D L A G Q L T D H D W S L F N S I H Q V E L I	645
S S K D L A Y Q M T I Y D W E L F N C V H R L E L I	797
A A H L R R Q K N V N S F F A V M F G L G K S P I S	725
A A H C R R Y K N L N S F F A I V X G L G K I A V S	876
M T F I H E G N H T L W E N L I N F G R M R N M A R	805
M T F T H E G N K T F I D N L V N F G R M R N I A N	956
V Q Q L K V I D N Q R E L S R L S R E L E P	881
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FIG. 3F-3

## SEQUENCE LISTING

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Housman, David

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gctctgagag tgtaacttgg gtctagccca ctggcacccgg cagcc atg gcg agc act 177  
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Arg Met Phe Leu Met Met His Pro Trp Tyr Ile Pro Ser Ser Gln Leu  
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Asn Ser Leu Gln Val Lys Thr Cys His Leu Val Arg Tyr Trp Val Ser  
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Ala Phe Pro Ala Glu Phe Asp Leu Asn Pro Glu Leu Ala Glu Pro Ile  
85 90 95 100

aag gag ctg aag gct ctg tta gac caa gaa gga aac cgc agg cac agc 513  
Lys Glu Leu Lys Ala Leu Leu Asp Gln Glu Gly Asn Arg Arg His Ser  
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Ser Leu Ile Asp Ile Glu Ser Val Pro Thr Tyr Lys Trp Lys Arg Gln  
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 Gln Asn Gln Asp Gly Cys Ile Ser Arg Glu Glu Met Ile Ser Tyr Phe  
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Ser Ser Gln Leu Ala Ser Lys Leu Leu His Phe Tyr Gln Gln Ser Arg  
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Arg Arg His Ser Ser Leu Ile Asp Ile Glu Ser Val Pro Thr Tyr Lys  
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Trp Lys Arg Gln Val Thr Gln Arg Asn Pro Val Glu Gln Lys Lys Arg  
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Lys Met Ser Leu Leu Phe Asp His Leu Glu Pro Met Glu Leu Ala Glu  
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His Leu Thr Tyr Leu Glu Tyr Arg Ser Phe Cys Lys Ile Leu Phe Gln  
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Asp Tyr His Ser Phe Val Thr His Gly Cys Thr Val Asp Asn Pro Val  
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Leu Glu Arg Phe Ile Ser Leu Phe Asn Ser Val Ser Gln Trp Val Gln  
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Leu Met Ile Leu Ser Lys Pro Thr Ala Thr Gln Arg Ala Leu Val Ile  
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Tyr Arg Arg Arg Leu Ala Ala Cys Val Gly Phe Arg Phe Pro Ile Leu  
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Gly Val His Leu Lys Asp Leu Val Ala Leu Gln Leu Ala Leu Pro Asp  
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Trp Leu Asp Pro Gly Arg Thr Arg Leu Asn Gly Ala Lys Met Arg Gln.  
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Leu Phe Ser Ile Leu Glu Glu Leu Ala Met Val Thr Ser Leu Arg Pro  
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Pro Val Gln Ala Asn Pro Asp Leu Leu Ser Leu Leu Thr Val Ser Leu  
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Lys Leu Asp Gln Ala Leu Val Ala Glu His Ile Glu Lys Met Val Glu  
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Ser Val Phe Arg Asn Phe Asp Val Asp Gly Asp Gly His Ile Ser Gln  
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Gly Asp Leu Asp Gln Asn Gln Asp Gly Cys Ile Ser Arg Glu Glu Met  
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Ile Ser Tyr Phe Leu Arg Ser Ser Val Leu Gly Gly Arg Met Gly

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485

490

495

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Arg	His	Cys	Lys	Ala	Leu	Ile	Leu	Gly	Ile	Tyr	Lys	Gln	Gly	Leu	Lys
				515			520				525				
Cys	Arg	Ala	Cys	Gly	Val	Asn	Cys	His	Lys	Gln	Cys	Lys	Asp	Arg	Leu
					530		535				540				
Ser	Val	Glu	Cys	Arg	Arg	Arg	Ala	Gln	Ser	Val	Ser	Leu	Glu	Gly	Ser
					545		550			555				560	
Ala	Pro	Ser	Pro	Ser	Pro	Thr	His	Thr	His	His	Arg	Ala	Phe	Ser	Phe
					565			570					575		
Ser	Leu	Pro	Arg	Pro	Gly	Arg	Arg	Ser	Ser	Arg	Pro	Pro	Glu	Ile	Arg
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ggagcgcagc ctggcccaag cccaccccgc gccggcgccg atg gca ggc acc ctg 175  
Met Ala Gly Thr Leu  
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gac ctg gac aag ggc tgc acg gtg gag gag ctg ctc cgc ggg tgc atc 223  
 Asp Leu Asp Lys Gly Cys Thr Val Glu Glu Leu Leu Arg Gly Cys Ile  
           10             15             20

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 Ala Lys Leu Leu His Ile Tyr Gln Gln Ser Arg Lys Asp Asn Ser Asn  
       55                 60                 65

tcc	ctg	cag	gtg	aaa	acg	tgc	cac	ctg	gtc	agg	tac	tgg	atc	tcc	gcc	415
Ser	Leu	Gln	Val	Lys	Thr	Cys	His	Leu	Val	Arg	Tyr	Trp	Ile	Ser	Ala	
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Phe Pro Ala Glu Phe Asp Leu Asn Pro Glu Leu Ala Glu Gln Ile Lys
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gag ctg aag gct ctg cta gac caa gaa ggg aac cga cgg cac agc agc      511
Glu Leu Lys Ala Leu Leu Asp Gln Glu Gly Asn Arg Arg His Ser Ser
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ctt atc gac ata gac agc gtc cct acc tac aag tgg aag cggtt cag gtg 559  
 Leu Ile Asp Ile Asp Ser Val Pro Thr Tyr Lys Trp Lys Arg Gln Val  
     120             125             130

- 6 -

act cag cg <sup>g</sup> aac cct gt <sup>g</sup> gga cag aaa aag cgc aag atg tcc ctg ttg	607
Thr Gln Arg Asn Pro Val Gly Gln Lys Lys Arg Lys Met Ser Leu Leu	
135 140 145	
ttt gag cac ctg gag ccc atg gag ctg gc <sup>g</sup> gag cat ctc acc tac ttg	655
Phe Asp His Leu Glu Pro Met Glu Leu Ala Glu His Leu Thr Tyr Leu	
150 155 160 165	
gag tat cgc tcc ttc tgc aag atc ctg ttt cag gac tat cac agt ttc	703
Glu Tyr Arg Ser Phe Cys Lys Ile Leu Phe Gln Asp Tyr His Ser Phe	
170 175 180	
gt <sup>g</sup> act cat ggc tgc act gt <sup>g</sup> gac aac ccc gtc ctg gag cg <sup>g</sup> ttc atc	751
Val Thr His Gly Cys Thr Val Asp Asn Pro Val Leu Glu Arg Phe Ile	
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Ser Leu Phe Asn Ser Val Ser Gln Trp Val Gln Leu Met Ile Leu Ser	
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aaa ccc aca gcc ccg cag cg <sup>g</sup> gcc ctg gtc atc aca cac ttt gtc cac	847
Lys Pro Thr Ala Pro Gln Arg Ala Leu Val Ile Thr His Phe Val His	
215 220 225	
gt <sup>g</sup> g <sup>c</sup> gag aag ctg cta cag ctg cag aac ttc aac acg ctg atg gca	895
Val Ala Glu Lys Leu Glu Leu Gln Asn Phe Asn Thr Leu Met Ala	
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gt <sup>g</sup> gtc ggg ggc ctg agc cac agc tcc atc tcc cgc ctc aag gag acc	943
Val Val Gly Gly Leu Ser His Ser Ser Ile Ser Arg Leu Lys Glu Thr	
250 255 260	
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His Ser His Val Ser Pro Glu Thr Ile Lys Leu Trp Glu Gly Leu Thr	
265 270 275	
gaa cta gt <sup>g</sup> acg g <sup>c</sup> aca ggc aac tat ggc aac tac cg <sup>g</sup> cgt cg <sup>g</sup> ctg	1039
Glu Leu Val Thr Ala Thr Gly Asn Tyr Gly Asn Tyr Arg Arg Arg Leu	
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Ala Ala Cys Val Gly Phe Arg Phe Pro Ile Leu Gly Val His Leu Lys	
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Arg Thr Arg Leu Asn Gly Ala Lys Met Lys Gln Leu Phe Ser Ile Leu	
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Glu Glu Leu Ala Met Val Thr Ser Leu Arg Pro Pro Val Gln Ala Asn	
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Pro Asp Leu Leu Ser Leu Leu Thr Val Ser Leu Asp Gln Tyr Gln Thr	
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gag gat gag ctg tac cag ctg tcc ctg cag cg <sup>g</sup> gag cc <sup>g</sup> cgc tcc aag	1327
Glu Asp Glu Leu Tyr Gln Leu Ser Leu Gln Arg Glu Pro Arg Ser Lys	
375 380 385	
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Ser Ser Pro Thr Ser Pro Thr Ser Cys Thr Pro Pro Pro Arg Pro Pro	
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Leu Val Val Glu His Ile Glu Lys Met Val Glu Ser Val Phe Arg Asn	
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- 7 -

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Lys Asp Asn Ser Asn Ser Leu Gln Val Lys Thr Cys His Leu Val Arg 65 70 75 80	

- 8 -

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 Ser Val Phe Arg Asn Phe Asp Val Asp Gly Asp Gly His Ile Ser Gln  
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- 9 -

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- 11 -

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- 12 -

715

720

725

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- 13 -

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Lys Lys Arg Lys Val Ser Leu Leu Phe Asp His Leu Glu Pro Glu Glu		
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Asn Pro Thr Met Glu Arg Ser Ile Ala Leu Cys Asn Gly Ile Ser Gln		
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Trp Val Gln Leu Met Val Leu Ser Arg Pro Thr Pro Gln Leu Arg Ala		
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Glu Val Phe Ile Lys Phe Ile His Val Ala Gln Lys Leu His Gln Leu		
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Gln Asn Phe Asn Thr Leu Met Ala Val Ile Gly Gly Leu Cys His Ser		
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Ser Ile Ser Arg Leu Lys Glu Thr Ser Ser His Val Pro His Glu Ile		
305	310	315
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Asn Lys Val Leu Gly Glu Met Thr Glu Leu Leu Ser Ser Cys Arg Asn		
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Tyr Asp Asn Tyr Arg Arg Ala Tyr Gly Glu Cys Thr His Phe Lys Ile		
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Pro Ile Leu Gly Val His Leu Lys Asp Leu Ile Ser Leu Tyr Glu Ala		
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Met Pro Asp Tyr Leu Glu Asp Gly Lys Val Asn Val Gln Lys Leu Leu		
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Pro Pro Leu Asp Ala Asn Lys Asp Leu Val His Leu Leu Thr Leu Ser		
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Leu Asp Leu Tyr Tyr Thr Glu Asp Glu Ile Tyr Glu Leu Ser Tyr Ala		
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Gln	Glu	Thr	Thr	Tyr	Ile	Lys	Pro	Thr	Phe	Cys	Asp	Asn	Cys	Ala	Gly
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His	Ser	Glu	Glu	Ser	Lys	Asp	Arg	Thr	Ile	Met	Leu	Leu	Gly	Val	Ser
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Ser	Gln	Lys	Ile	Ser	Val	Arg	Leu	Lys	Arg	Thr	Val	Ala	His	Lys	Thr
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Tyr	Ala	Gln	Lys	Gln	Ile	Glu	Ser	Leu	Gln	Leu	Gly	Lys	Ser	Asn	His
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 Met Gly Thr Leu  
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 Gly Lys Ala Arg Glu Ala Pro Arg Lys Pro Ser His Gly Cys Arg Ala  
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 Ala Ser Lys Ala Arg Leu Glu Ala Lys Pro Ala Asn Ser Pro Phe Pro 211  
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 Ser His Pro Ser Leu Ala His Ile Thr Gln Phe Arg Met Met Val Ser  
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- 18 -

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- 20 -

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Met Val Leu Lys Arg Met His Arg Pro Arg Cys Cys
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Ile Leu Ala Leu Gly Leu Gly Val His Ser Arg Ser Gln Ala Val Gly  
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Pro Glu Pro Gln Pro Ala Gly Thr His Asp Val Glu Glu Glu Leu Val  
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gag ctg tgt ctc tgc ccc gtg ccc ggc ccc cggt gcc cag ctg ctc aaa Glu Leu Cys Leu Cys Pro Val Pro Gly Pro Arg Ala Gln Leu Leu Lys 680 685 690			2297
aag ttc att aag ctg gcg gcc cac ctc aag gag cag aag aat gtc aat Lys Phe Ile Lys Leu Ala Ala His Leu Lys Glu Gln Lys Asn Val Asn 695 700 705 710			2345
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cta gcc cac acc tgg gag cgg ctg cct cac aaa gtc cgg aag ctg tac Leu Ala His Thr Trp Glu Arg Leu Pro His Lys Val Arg Lys Leu Tyr 730 735 740			2441
tcc gcc ctc gag agg ctg ctg gat ccc tca tgg aac cac cgg gta tac Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser Trp Asn His Arg Val Tyr 745 750 755			2489
cga ctg gcc ctc gcc aag ctc tcc cct cct gtc atc ccc ttc atg ccc Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro Val Ile Pro Phe Met Pro 760 765 770			2537
ctt ctt ctc aaa gac atg acc ttc att cat gag gga aac cac aca cta Leu Leu Leu Lys Asp Met Thr Phe Ile His Glu Gly Asn His Thr Leu 775 780 785 790			2585
gtg gag aat ctc atc aac ttt gag aag atg aga atg atg gtc aga gcc Val Glu Asn Leu Ile Asn Phe Glu Lys Met Arg Met Met Ala Arg Ala 795 800 805			2633
gcg cgg atg ctg cac cac tgc cga agc cac aac cct gtg cct ctc tca Ala Arg Met Leu His His Cys Arg Ser His Asn Pro Val Pro Leu Ser 810 815 820			2681
cca ctc aga agc cga gtt tcc cac ctc cac gag gac agc cag gtg gcg Pro Leu Arg Ser Arg Val Ser His Leu His Glu Asp Ser Gln Val Ala 825 830 835			2729
agg att tcc aca tgc tcg gag cag tcc ctg agc acc cgg agt cca gcc Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu Ser Thr Arg Ser Pro Ala 840 845 850			2777
agc acc tgg gct tat gtc cag cag ctg aag gtc att gac aac cag cgg Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys Val Ile Asp Asn Gln Arg 855 860 865 870			2825
gaa ctc tcc cgc ctg tcc cga gag ctg gag cca tgaggaggg ctggactgg			2878

- 30 -

Glu Leu Ser Arg Leu Ser Arg Glu Leu Glu Pro	
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gtggaggcag ccatccccctg tgatgactgg cagctaagga ggacctcgga gtggaccaaa	3058
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<213> Homo sapiens

Met Val Leu Arg Arg Met His Arg Pro Arg Ser Cys Ser Tyr Gln Leu																																																																																																																											
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10	15																																																																																																																										
Leu Leu Glu His Gln His Pro Ser Cys Ile Gln Gly Leu Arg Trp Thr																																																																																																																											
20	25	30				Pro Leu Thr Asn Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu		35	40	45				Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Gln		50	55	60				His Leu Leu Ala Thr Cys Pro Asn Leu Ile Arg Asp Arg Lys Tyr His		65	70	75	80			Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly		85	90	95				Ile Leu Ala Leu Gly Val His Ser Arg Ser Gln Val Val Gly		100	105	110				Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His		115	120	125				Asp Trp Ala Phe Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly		130	135	140				Pro Glu Pro Glu Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala		145	150	155	160			Glu Ala Val Ala Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr		165	170	175				Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp		180	185	190				Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser		195	200	205				Asn Ser Val Lys Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His		210	215	220				Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser		225	230	235	240			Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys		245	250	255									
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Pro Leu Thr Asn Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu																																																																																																																											
35	40	45				Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Gln		50	55	60				His Leu Leu Ala Thr Cys Pro Asn Leu Ile Arg Asp Arg Lys Tyr His		65	70	75	80			Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly		85	90	95				Ile Leu Ala Leu Gly Val His Ser Arg Ser Gln Val Val Gly		100	105	110				Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His		115	120	125				Asp Trp Ala Phe Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly		130	135	140				Pro Glu Pro Glu Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala		145	150	155	160			Glu Ala Val Ala Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr		165	170	175				Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp		180	185	190				Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser		195	200	205				Asn Ser Val Lys Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His		210	215	220				Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser		225	230	235	240			Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys		245	250	255																	
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Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Gln																																																																																																																											
50	55	60				His Leu Leu Ala Thr Cys Pro Asn Leu Ile Arg Asp Arg Lys Tyr His		65	70	75	80			Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly		85	90	95				Ile Leu Ala Leu Gly Val His Ser Arg Ser Gln Val Val Gly		100	105	110				Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His		115	120	125				Asp Trp Ala Phe Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly		130	135	140				Pro Glu Pro Glu Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala		145	150	155	160			Glu Ala Val Ala Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr		165	170	175				Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp		180	185	190				Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser		195	200	205				Asn Ser Val Lys Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His		210	215	220				Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser		225	230	235	240			Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys		245	250	255																									
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His Leu Leu Ala Thr Cys Pro Asn Leu Ile Arg Asp Arg Lys Tyr His																																																																																																																											
65	70	75	80			Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly		85	90	95				Ile Leu Ala Leu Gly Val His Ser Arg Ser Gln Val Val Gly		100	105	110				Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His		115	120	125				Asp Trp Ala Phe Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly		130	135	140				Pro Glu Pro Glu Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala		145	150	155	160			Glu Ala Val Ala Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr		165	170	175				Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp		180	185	190				Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser		195	200	205				Asn Ser Val Lys Arg Glu Leu Ala Ala Val Leu Leu Phe Glu Pro His		210	215	220				Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser		225	230	235	240			Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys		245	250	255																																	
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Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly																																																																																																																											
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Ile Leu Ala Leu Gly Val His Ser Arg Ser Gln Val Val Gly																																																																																																																											
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Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His																																																																																																																											
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Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp																																																																																																																											
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Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser																																																																																																																											
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- 31 -

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 275 280 285  
 Asn Cys His Phe Leu Arg Val Asp Lys Gln Asp Phe Asn Arg Ile Ile  
 290 295 300  
 Lys Asp Val Glu Ala Lys Thr Met Arg Leu Glu Glu His Gly Lys Val  
 305 310 315 320  
 Val Leu Val Leu Glu Arg Ala Ser Gln Gly Ala Gly Pro Ser Arg Pro  
 325 330 335  
 Pro Thr Pro Gly Arg Asn Arg Tyr Thr Val Met Ser Gly Thr Pro Asp  
 340 345 350  
 Lys Ile Leu Glu Leu Leu Glu Ala Met Gly Leu Asp Ser Ser Ala  
 355 360 365  
 His Asp Pro Lys Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg  
 370 375 380  
 Val Phe Met Pro Ser Ala Gln Leu Cys Ala Ala Leu Leu His His Phe  
 385 390 395 400  
 His Val Glu Pro Ala Gly Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val  
 405 410 415  
 Cys Asn Lys Arg Gln Gln Ile Leu Arg Leu Val Ser Gln Trp Val Ala  
 420 425 430  
 Leu Tyr Gly Ser Met Leu His Thr Asp Pro Val Ala Thr Ser Phe Leu  
 435 440 445  
 His Lys Leu Ser Asp Leu Val Gly Arg Asp Thr Arg Leu Ser Asn Leu  
 450 455 460  
 Leu Arg Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn  
 465 470 475 480  
 Gly Cys Gly Asn Ala Ser Pro Gln Met Lys Ala Arg Asn Leu Pro Val  
 485 490 495  
 Trp Leu Pro Asn Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile  
 500 505 510  
 Gln Val Gly Asp Lys Val Pro Tyr Asp Ile Cys Arg Pro Asp His Ser  
 515 520 525  
 Val Leu Thr Leu Gln Leu Pro Val Thr Ala Ser Val Arg Glu Val Met  
 530 535 540  
 Ala Ala Leu Ala Gln Glu Asp Gly Trp Thr Lys Gly Gln Val Leu Val  
 545 550 555 560  
 Lys Val Asn Ser Ala Gly Asp Ala Ile Gly Leu Gln Pro Asp Ala Arg  
 565 570 575  
 Gly Val Ala Thr Ser Leu Gly Leu Asn Glu Arg Leu Phe Val Val Asn  
 580 585 590  
 Pro Gln Glu Val His Glu Leu Ile Pro His Pro Asp Gln Leu Gly Pro  
 595 600 605  
 Thr Val Gly Ser Ala Glu Gly Leu Asp Leu Val Ser Ala Lys Asp Leu  
 610 615 620  
 Ala Gly Gln Leu Thr Asp His Asp Trp Ser Leu Phe Asn Ser Ile His  
 625 630 635 640  
 Gln Val Glu Leu Ile His Tyr Val Leu Gly Pro Gln His Leu Arg Asp  
 645 650 655

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Val Thr Thr Ala Asn Leu Glu Arg Phe Met Arg Arg Phe Asn Glu Leu  
 660 665 670

Gln Tyr Trp Val Ala Thr Glu Leu Cys Leu Cys Pro Val Pro Gly Pro  
 675 680 685

Arg Ala Gln Leu Leu Lys Lys Phe Ile Lys Leu Ala Ala His Leu Lys  
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Glu Gln Lys Asn Val Asn Ser Phe Phe Ala Val Met Phe Gly Leu Ser  
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Asn Ser Pro Ile Ser Arg Leu Ala His Thr Trp Glu Arg Leu Pro His  
 725 730 735

Lys Val Arg Lys Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser  
 740 745 750

Trp Asn His Arg Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro  
 755 760 765

Val Ile Pro Phe Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His  
 770 775 780

Glu Gly Asn His Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met  
 785 790 795 800

Arg Met Met Ala Arg Ala Ala Arg Met Leu His His Cys Arg Ser His  
 805 810 815

Asn Pro Val Pro Leu Ser Pro Leu Arg Ser Arg Val Ser His Leu His  
 820 825 830

Glu Asp Ser Gln Val Ala Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu  
 835 840 845

Ser Thr Arg Ser Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys  
 850 855 860

Val Ile Asp Asn Gln Arg Glu Leu Ser Arg Leu Ser Arg Glu Leu Glu  
 865 870 875 880

Pro

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 <213> Homo sapiens

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 ggcaggtggg cctggctgtg gaggatagcc cagctctggg agcacccggg gtgggagccc 180  
 tccctgacgt ggtgccggag gggacactac tcaac atg gtg ttg aga agg atg 233  
 Met Val Leu Arg Arg Met  
 1 5

cac cgg ccc cga agc tgc tcc tac cag ctg ctg gag cac cag cat 281  
 His Arg Pro Arg Ser Cys Ser Tyr Gln Leu Leu Leu Glu His Gln His  
 10 15 20

ccg agc tgc atc cag ggg ctg cgc tgg aca cca ctc acc aac agc gag 329  
 Pro Ser Cys Ile Gln Gly Leu Arg Trp Thr Pro Leu Thr Asn Ser Glu  
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- 33 -

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tgc tgc tct ggc cgg gag ctg gtg gat ggg atc ttg gcc ctg gga ctt Cys Cys Ser Gly Arg Glu Leu Val Asp Gly Ile Leu Ala Leu Gly Leu 90 95 100	521
ggg gtc cat tcc cgg agc caa gtt gtg gga atc tgc cag gtg ctg ctg Gly Val His Ser Arg Ser Gln Val Val Gly Ile Cys Gln Val Leu Leu 105 110 115	569
gat gaa ggt gcc ctc tgc cat gtg aaa cac gac tgg gcc ttc cag gac Asp Glu Gly Ala Leu Cys His Val Lys His Asp Trp Ala Phe Gln Asp 120 125 130	617
cga gat gcc caa ttc tac cgg ttc ccc ggg ccc gag ccc gag ccc gtg Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly Pro Glu Pro Glu Pro Val 135 140 145 150	665
gga act cat gag atg gag gag ttg gcc gaa gct gtg gcc ctg ctc Gly Thr His Glu Met Glu Glu Leu Ala Glu Ala Val Ala Leu Leu 155 160 165	713
tcc cag cgg ggg cct gac gcc ctg ctc act gtg gca ctt cga aag ccc Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr Val Ala Leu Arg Lys Pro 170 175 180	761
cca ggt cag cgc acg gat gaa gag ctg gac ctc atc ttt gag gag ctg Pro Gly Gln Arg Thr Asp Glu Leu Asp Leu Ile Phe Glu Glu Leu 185 190 195	809
ctg cac atc aag gct gtg gcc cac ctc tcc aac tcg gtg aag cga gaa Leu His Ile Lys Ala Val Ala His Leu Ser Asn Ser Val Lys Arg Glu 200 205 210	857
tta gcg gct gtt ctg ctc ttt gaa cca cac agc aag gca ggg acc gtg Leu Ala Ala Val Leu Leu Phe Glu Pro His Ser Lys Ala Gly Thr Val 215 220 225 230	905
ttg ttc agc cag ggg gac aag ggc act tcg tgg tac att atc tgg aag Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser Trp Tyr Ile Ile Trp Lys 235 240 245	953
gga tct gtc aac gtg gtg acc cat ggc aag ggg ctg gtg acc acc ctg Gly Ser Val Asn Val Val Thr His Gly Lys Gly Leu Val Thr Thr Leu 250 255 260	1001
cat gag gga gat gat ttt gga cag ctg gct ctg gtg aat gat gca ccc His Glu Gly Asp Asp Phe Gly Gln Leu Ala Leu Val Asn Asp Ala Pro 265 270 275	1049
cgg gca gcc acc atc atc ctg cga gaa tac aac tgt cat ttc ctg cgt Arg Ala Ala Thr Ile Ile Leu Arg Glu Tyr Asn Cys His Phe Leu Arg 280 285 290	1097
gtg gac aag cag gac ttc aac cgt atc atc aag gat gtg gag gca aag Val Asp Lys Gln Asp Phe Asn Arg Ile Ile Lys Asp Val Glu Ala Lys 295 300 305 310	1145
acc atg cgg ctg gaa gaa cat ggc aaa gtg gtg ctg gtg ctg gag aga Thr Met Arg Leu Glu Glu His Gly Lys Val Val Leu Val Leu Glu Arg 315 320 325	1193
gcc tct cag ggc gcc ggc cct tcc cga ccc cca acc cca ggc agg aac Ala Ser Gln Gly Ala Gly Pro Ser Arg Pro Pro Thr Pro Gly Arg Asn	1241

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330

335

340

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cggtatacagtgatgtctggcactccagataaatgcataaggcttctg 1289
Arg Tyr Thr Val Met Ser Gly Thr Pro Asp Lys Ile Leu Glu Leu Leu
            345          350          355

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 Leu Glu Ala Met Gly Leu Asp Ser Ser Ala His Asp Pro Lys Glu Thr  
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ttc ctc agc gac ttc ctc ctg acc cac agg gtc ttc atg ccc agc gcc 1385  
 Phe Leu Ser Asp Phe Leu Thr His Arg Val Phe Met Pro Ser Ala  
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caa ctc tgc gct gcc ctt ctg cac cac ttc cat gtg gag cct gcg ggt 1433  
 Gln Leu Cys Ala Ala Leu Leu His His Phe His Val Glu Pro Ala Gly  
 395 400 405

ggc agc gag cag gag cg<sub>c</sub> agc acc tac gtc tgc aac aag agg cag cag 1481  
 Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val Cys Asn Lys Arg Gln Gln  
 410 415 420

atc ttg cggtcg agc cag tgg gtg gcc ctg tat ggc tcc atg ctc 1529  
 Ile Leu Arg Leu Val Ser Gln Trp Val Ala Leu Tyr Gly Ser Met Leu  
 425 430 435

cac act gac cct gtg gcc acc agc ttc ctc cag aaa ctc tca gac ctg 1577  
 His Thr Asp Pro Val Ala Thr Ser Phe Leu Gln Lys Leu Ser Asp Leu  
 440 445 450

gtg ggc agg gac acc cga ctc agc aac ctg ctg agg gag gag cag tgg cca 1625  
 Val Gly Arg Asp Thr Arg Leu Ser Asn Leu Leu Arg Glu Gln Trp Pro  
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gag agg cgg cga tgc cac agg ttg gag aat ggc tgt ggg aat gca tct 1673  
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cct cag atg aag gtg tct gcc tgg ccc cag ttt ctt tcc tcc tct gct cct 1721  
 Pro Gln Met Lys Val Ser Ala Trp Pro Gln Phe Leu Ser Ser Ala Pro  
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cct gga ctg cag gca cct cct tcg ccc cct gac cct gag ggg ctc tgt 1769  
 Pro Gly Leu Gln Ala Pro Pro Ser Pro Pro Asp Pro Glu, Gly Leu Cys  
 505 510 515

ggg cgt ggg aag ctc tcc tcc cac aga cac acc ctt ggg tct ctg ata 1817  
 Gly Arg Gly Lys Leu Ser Ser His Arg His Thr Leu Gly Ser Leu Ile  
 520 525 530

ggt gtt cac ggg gcc ctt gct gca tgt ggt gcc ctg ggc cag gcc gtg 1865  
 Gly Val His Gly Ala Leu Ala Ala Cys Gly Ala Leu Gly Gln Ala Val  
 535 540 545 550

cca gga ggc gca gag gcc taagggtggcc tcccttcctcg cccactccct  
Pro Gly Gly Ala Glu Ala  
555

gactcaatgg gccttttatt ccttttgaaa ggtaattcat gcccacaggt agagcctggg 1973

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ttctctggct ctacccggca ggatccttct catttcatac tcaggccaaat gctgggttcc 2453

- 35 -

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<213> *Homo sapiens*

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 Pro Leu Thr Asn Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu  
 35 40 45

Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Gln  
50 55 60

- 36 -

His	Leu	Leu	Ala	Thr	Cys	Pro	Asn	Leu	Ile	Arg	Asp	Arg	Lys	Tyr	His
65						70				75					80
Leu	Arg	Leu	Tyr	Arg	Gln	Cys	Cys	Ser	Gly	Arg	Glu	Leu	Val	Asp	Gly
	85						90							95	
Ile	Leu	Ala	Leu	Gly	Leu	Gly	Val	His	Ser	Arg	Ser	Gln	Val	Val	Gly
	100						105						110		
Ile	Cys	Gln	Val	Leu	Leu	Asp	Glu	Gly	Ala	Leu	Cys	His	Val	Lys	His
	115					120					125				
Asp	Trp	Ala	Phe	Gln	Asp	Arg	Asp	Ala	Gln	Phe	Tyr	Arg	Phe	Pro	Gly
	130					135					140				
Pro	Glu	Pro	Glu	Pro	Val	Gly	Thr	His	Glu	Met	Glu	Glu	Glu	Leu	Ala
	145				150				155					160	
Glu	Ala	Val	Ala	Leu	Leu	Ser	Gln	Arg	Gly	Pro	Asp	Ala	Leu	Leu	Thr
						165		170					175		
Val	Ala	Leu	Arg	Lys	Pro	Pro	Gly	Gln	Arg	Thr	Asp	Glu	Glu	Leu	Asp
				180			185					190			
Leu	Ile	Phe	Glu	Glu	Leu	Leu	His	Ile	Lys	Ala	Val	Ala	His	Leu	Ser
	195					200		205							
Asn	Ser	Val	Lys	Arg	Glu	Leu	Ala	Ala	Val	Leu	Phe	Glu	Pro	His	
	210					215					220				
Ser	Lys	Ala	Gly	Thr	Val	Leu	Phe	Ser	Gln	Gly	Asp	Lys	Gly	Thr	Ser
	225				230				235				240		
Trp	Tyr	Ile	Ile	Trp	Lys	Gly	Ser	Val	Asn	Val	Val	Thr	His	Gly	Lys
					245			250				255			
Gly	Leu	Val	Thr	Thr	Leu	His	Glu	Gly	Asp	Asp	Phe	Gly	Gln	Leu	Ala
				260			265					270			
Leu	Val	Asn	Asp	Ala	Pro	Arg	Ala	Ala	Thr	Ile	Ile	Leu	Arg	Glu	Tyr
				275			280					285			
Asn	Cys	His	Phe	Leu	Arg	Val	Asp	Lys	Gln	Asp	Phe	Asn	Arg	Ile	Ile
	290				295						300				
Lys	Asp	Val	Glu	Ala	Lys	Thr	Met	Arg	Leu	Glu	Glu	His	Gly	Lys	Val
	305				310				315				320		
Val	Leu	Val	Leu	Glu	Arg	Ala	Ser	Gln	Gly	Ala	Gly	Pro	Ser	Arg	Pro
					325			330				335			
Pro	Thr	Pro	Gly	Arg	Asn	Arg	Tyr	Thr	Val	Met	Ser	Gly	Thr	Pro	Asp
				340			345					350			
Lys	Ile	Leu	Glu	Leu	Leu	Glu	Ala	Met	Gly	Leu	Asp	Ser	Ser	Ala	
				355			360				365				
His	Asp	Pro	Lys	Glu	Thr	Phe	Leu	Ser	Asp	Phe	Leu	Leu	Thr	His	Arg
	370				375						380				
Val	Phe	Met	Pro	Ser	Ala	Gln	Leu	Cys	Ala	Ala	Leu	Leu	His	His	Phe
	385				390				395				400		
His	Val	Glu	Pro	Ala	Gly	Gly	Ser	Glu	Gln	Glu	Arg	Ser	Thr	Tyr	Val
				405			410					415			
Cys	Asn	Lys	Arg	Gln	Gln	Ile	Leu	Arg	Leu	Val	Ser	Gln	Trp	Val	Ala
				420			425					430			
Leu	Tyr	Gly	Ser	Met	Leu	His	Thr	Asp	Pro	Val	Ala	Thr	Ser	Phe	Leu
				435			440					445			
Gln	Lys	Leu	Ser	Asp	Leu	Val	Gly	Arg	Asp	Thr	Arg	Leu	Ser	Asn	Leu
				450			455					460			

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Leu Arg Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn  
 465                    470                    475                    480

Gly Cys Gly Asn Ala Ser Pro Gln Met Lys Val Ser Ala Trp Pro Gln  
 485                    490                    495

Phe Leu Ser Ser Ala Pro Pro Gly Leu Gln Ala Pro Pro Ser Pro Pro  
 500                    505                    510

Asp Pro Glu Gly Leu Cys Gly Arg Gly Lys Leu Ser Ser His Arg His  
 515                    520                    525

Thr Leu Gly Ser Leu Ile Gly Val His Gly Ala Leu Ala Ala Cys Gly  
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<212> DNA

<213> Rattus norvegicus

<220>

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1                5                    10                    15	

att aat gga cgc ctg ttt gcc tgc ccg cga gag caa ttc gac tca ctg	95
Ile Asn Gly Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu	
20                25                    30	

act ccc ttg cca gaa cag gag ggc ccg acc act ggg aca gtg ggg acg	143
Thr Pro Leu Pro Glu Gln Glu Gly Pro Thr Thr Gly Thr Val Gly Thr	
35                40                    45	

ttt gaa ctg atg agc tcg aaa gac ttg gcg tac cag atg aca acg tat	191
Phe Glu Leu Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Thr Tyr	
50                55                    60	

gac tgg gaa ctc ttc aac tgt gtc cag gag ctg gag cta atc tac cac	239
Asp Trp Glu Leu Phe Asn Cys Val Leu Glu Leu Glu Leu Ile Tyr His	
65                70                    75	

aca ttt gga agg cat aat ttt aaa aag acc aca gca aac ttg gat ttg	287
Thr Phe Gly Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu	
80                85                    90                    95	

ttc ctg agg aga ttt aat gaa att cag ttt tgg gtt gtc act gag atc	335
Phe Leu Arg Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile	
100                105                    110	

tgc ctt tgt tcc cag ctc agc aag cgt gtt cag ctt ttg aaa aaa tgt	383
Cys Leu Cys Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Cys	
115                120                    125	

atc aag ata gcg gct cac tgc aag gag tac aaa aac ttg aat tcc ttc	431
Ile Lys Ile Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe	
130                135                    140	

ttc ggc atc gtc atg ggg ctc agt aac gtt gct gag agc cgc ctg gca	479
Phe Gly Ile Val Met Gly Leu Ser Asn Val Ala Glu Ser Arg Leu Ala	
145                150                    155	

tta aca tgg gag aaa ctg ccg agc aag ttt aag aag ttc tat gcg gag	527
Leu Thr Trp Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu	
160                165                    170                    175	

- 38 -

ttt gag agc tta atg gat cct tcc aga aat cac aag gcg tac agg ctg Phe Glu Ser Leu Met Asp Pro Ser Arg Asn His Lys Ala Tyr Arg Leu	575
180 185 190	
aca gca gct aaa ctg gag ccc ccc ctc atc cct ttc atg ccc ttg ctt Thr Ala Ala Lys Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu	623
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210 215 220	
aat cta gta aac ttt gaa aaa atg cgc atg att gca aat act gcc aga Asn Leu Val Asn Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg	719
225 230 235	
acg gtg cgc tac tac agg agc cag cca ttc aat ccg gat gct gct caa Thr Val Arg Tyr Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln	767
240 245 250 255	
gct aat aag aac cat cag gat gtc cgg agt tat gta cgg caa tta aat Ala Asn Lys Asn Gln Asp Val Arg Ser Tyr Val Arg Gln Leu Asn	815
260 265 270	
gtg att gac aac cag aga act tta tca cag atg tca cac aga tta gag Val Ile Asp Asn Gln Arg Thr Leu Ser Gln Met Ser His Arg Leu Glu	863
275 280 285	
cct cgc agg cca tagacatctg cagtccccag agtgatgctc cgtctccagt Pro Arg Arg Pro	915
290	
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tcattaaata tacttgacaa gaaaaaaaaa a	1966

<210> 16  
<211> 291  
<212> PRT  
<213> Rattus norvegicus

<400> 16

- 39 -

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 Asn Gly Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu Thr  
 20 25 30  
 Pro Leu Pro Glu Gln Glu Gly Pro Thr Thr Gly Thr Val Gly Thr Phe  
 35 40 45  
 Glu Leu Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Thr Tyr Asp  
 50 55 60  
 Trp Glu Leu Phe Asn Cys Val Leu Glu Leu Glu Leu Ile Tyr His Thr  
 65 70 75 80  
 Phe Gly Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu Phe  
 85 90 95  
 Leu Arg Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile Cys  
 100 105 110  
 Leu Cys Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Cys Ile  
 115 120 125  
 Lys Ile Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe  
 130 135 140  
 Gly Ile Val Met Gly Leu Ser Asn Val Ala Glu Ser Arg Leu Ala Leu  
 145 150 155 160  
 Thr Trp Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe  
 165 170 175  
 Glu Ser Leu Met Asp Pro Ser Arg Asn His Lys Ala Tyr Arg Leu Thr  
 180 185 190  
 Ala Ala Lys Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu Ile  
 195 200 205  
 Lys Asp Met Thr Phe Thr His Glu Gly Asn Lys Thr Phe Ile Asp Asn  
 210 215 220  
 Leu Val Asn Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg Thr  
 225 230 235 240  
 Val Arg Tyr Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln Ala  
 245 250 255  
 Asn Lys Asn His Gln Asp Val Arg Ser Tyr Val Arg Gln Leu Asn Val  
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 Arg Arg Pro  
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 aggaaataac attatttcgc cagggtgata tggAACAAAC tggatatgtcg cctggcaggg 180

- 40 -

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ctggaaattt ggacggccctt tggagagtcc attctggaca acacaccccg ccatgcaacc	300		
atcgttacca gggagagcag tgaactgctc cgcatcgagc agaaggactt caaggcacta	360		
tggagaaaat atcgacagta tatggcagga ctctggctc ctccctt atg gta tta	415		
Met Val Leu			
1			
tgg aaa cgg gct cta aca atg aca gga ttc ctg aca agg aga aca cac	463		
Trp Lys Arg Ala Leu Thr Met Thr Gly Phe Leu Thr Arg Arg Thr His			
5	10		
ctc att gaa cct cac gtt cct ctt cgt cct gct aac acc att acc aag	511		
Leu Ile Glu Pro His Val Pro Leu Arg Pro Ala Asn Thr Ile Thr Lys			
20	25	30	35
gtc cct tca gag aag atc ctc aga gct gga aaa att tta cga aat gcc	559		
Val Pro Ser Glu Lys Ile Leu Arg Ala Gly Lys Ile Leu Arg Asn Ala			
40	45	50	
att ctc tct cga gca cct cac atg ata aga gat aga aaa tac cac cta	607		
Ile Leu Ser Arg Ala Pro His Met Ile Arg Asp Arg Lys Tyr His Leu			
55	60	65	
aag aca tac aga caa tgc tgt gtg gga act gaa ctg gtg gac tgg atg	655		
Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr Glu Leu Val Asp Trp Met			
70	75	80	
atc gac gag aca cca tgt gtt cac tcc cgg act caa gct gtt ggc atg	703		
Ile Asp Glu Thr Pro Cys Val His Ser Arg Thr Gln Ala Val Gly Met			
85	90	95	
tgg caa gtc ctg tta gaa gat ggt gtt ctc aac cac gtg gac cag gag	751		
Trp Gln Val Leu Leu Glu Asp Gly Val Leu Asn His Val Asp Gln Glu			
100	105	110	115
cac cat ttc caa gac ttt tat tta ttc tat cga ttt ctg gat gat gag	799		
His His Phe Gln Asp Phe Tyr Leu Phe Tyr Arg Phe Leu Asp Asp Glu			
120	125	130	
cac gag gat gcc cct ttg cct act gag gag gag aag aag gag tgg gat	847		
His Glu Asp Ala Pro Leu Pro Thr Glu Glu Lys Lys Glu Cys Asp			
135	140	145	
gag gag ctc cag gac acc atg ctg ctg tca cag atg ggc ccc gac	895		
Glu Glu Leu Gln Asp Thr Met Leu Leu Ser Gln Met Gly Pro Asp			
150	155	160	
gcc cac atg agg atg atc ctt cgc aaa cca cct ggc cag agg act gtg	943		
Ala His Met Arg Met Ile Leu Arg Lys Pro Pro Gly Gln Arg Thr Val			
165	170	175	
gat gac cta gag att atc tat gag gag ctt ctt cat att aaa gcc tta	991		
Asp Asp Leu Glu Ile Ile Tyr Glu Glu Leu Leu His Ile Lys Ala Leu			
180	185	190	195
tcc cat ctt tct acc aca gtg aaa cga gag tta gca ggt gtt ctc att	1039		
Ser His Leu Ser Thr Thr Val Lys Arg Glu Leu Ala Gly Val Leu Ile			
200	205	210	
ttt gag tct cac gcc aaa gga ggg act gtg ttg ttt aac cag ggg gaa	1087		
Phe Glu Ser His Ala Lys Gly Gly Thr Val Leu Phe Asn Gln Gly Glu			
215	220	225	
gaa ggt acc tcc tgg tac att att cta aaa gga tca gtg aat gta gtc	1135		
Glu Gly Thr Ser Trp Tyr Ile Ile Leu Lys Gly Ser Val Asn Val Val			
230	235	240	
att tac ggc aag ggt gtg gtc tgc acc ctg cat gaa gga gat gac ttc	1183		
Ile Tyr Gly Lys Gly Val Val Cys Thr Leu His Glu Gly Asp Asp Phe			
245	250	255	
ggc aag tta gca cta gtg aat gat gcc cca cga gct gcc tct atc gtc	1231		

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Gly Lys Leu Ala Leu Val Asn Asp Ala Pro Arg Ala Ala Ser Ile Val	260                    265                    270                    275	
tta cga gaa gat aac tgc cat ttc tta aga gta gac aag gag gat ttc		1279
Leu Arg Glu Asp Asn Cys His Phe Leu Arg Val Asp Lys Glu Asp Phe	280                    285                    290	
aac cgg atc cta agg gac gtg gag gcg aat aca gtc aga ctt aaa gaa		1327
Asn Arg Ile Leu Arg Asp Val Glu Ala Asn Thr Val Arg Leu Lys Glu	295                    300                    305	
cat gac caa gat gtc ttg gtg ctg gag aag gtc cca gca ggg aac aga		1375
His Asp Gln Asp Val Leu Val Leu Glu Lys Val Pro Ala Gly Asn Arg	310                    315                    320	
gct tct aat caa gga aac tca cag cct cag caa aag tat act gtg atg		1423
Ala Ser Asn Gln Gly Asn Ser Gln Pro Gln Gln Lys Tyr Thr Val Met	325                    330                    335	
tca gga aca cct gaa aaa att tta gag cat ttt cta gaa aca ata cgc		1471
Ser Gly Thr Pro Glu Lys Ile Leu Glu His Phe Leu Glu Thr Ile Arg	340                    345                    350                    355	
ctt gag gca act tta aat gaa gca aca gat tct gtt tta aat gac ttt		1519
Leu Glu Ala Thr Leu Asn Glu Ala Thr Asp Ser Val Leu Asn Asp Phe	360                    365                    370	
att atg atg cac tgt gtt ttt atg cca aat acc cag ctt tgc ccg gca		1567
Ile Met Met His Cys Val Phe Met Pro Asn Thr Gln Leu Cys Pro Ala	375                    380                    385	
ctg gtg gcc cac tac cac gca cag cct tca caa ggt aca gaa cag gag		1615
Leu Val Ala His Tyr His Ala Gln Pro Ser Gln Gly Thr Glu Gln Glu	390                    395                    400	
aaa atg gat tat gcc ctc aac aat aag agg cga gtc atc cgc ctg gtt		1663
Lys Met Asp Tyr Ala Leu Asn Asn Lys Arg Arg Val Ile Arg Leu Val	405                    410                    415	
cta cag tgg gct gcc atg tat gga gac ctc ctg caa gag gat gac gta		1711
Leu Gln Trp Ala Ala Met Tyr Gly Asp Leu Leu Gln Glu Asp Asp Val	420                    425                    430                    435	
tct atg gcc ttc ctg gag gag ttt tat gta tct gta tca gat gat gcc		1759
Ser Met Ala Phe Leu Glu Glu Phe Tyr Val Ser Val Ser Asp Asp Ala	440                    445                    450	
cgg atg att gct gcc ctc aag gag caa ctg cca gag ttg gag aag att		1807
Arg Met Ile Ala Ala Leu Lys Glu Gln Leu Pro Glu Leu Glu Lys Ile	455                    460                    465	
gtc aag caa atc tca gaa gat gca aag gca cca caa aag aag cac aag		1855
Val Lys Gln Ile Ser Glu Asp Ala Lys Ala Pro Gln Lys Lys His Lys	470                    475                    480	
gtt ctt ttg caa cag ttc aat acg ggc gat gag aga gcc cag aag cgc		1903
Val Leu Leu Gln Gln Phe Asn Thr Gly Asp Glu Arg Ala Gln Lys Arg	485                    490                    495	
cag cct atc cgc ggc tct gat gaa gtt ctg ttt aag gtc tat tgc atg		1951
Gln Pro Ile Arg Gly Ser Asp Glu Val Leu Phe Lys Val Tyr Cys Met	500                    505                    510                    515	
gac cac acc tac aca acc att cgg gtg cca gtg gcc act tcg gtg aag		1999
Asp His Thr Tyr Thr Ile Arg Val Pro Val Ala Thr Ser Val Lys	520                    525                    530	
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Ile Ile Val Lys Met Ser Ser Gly Gly Glu Lys Val Val Leu Lys Pro	550                    555                    560	

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aat gat gtt tca gta ttt acg acg ctc acc att aat gga cgc ctg ttt Asn Asp Val Ser Val Phe Thr Thr Leu Thr Ile Asn Gly Arg Leu Phe 565 570 575	2143
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tgt aag gag tat aaa aat ctg aat tcc ttt ttt gcc atc gtc atg gga Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe Ala Ile Val Met Gly 695 700 705	2527
cta agt aac att gct gtg agc cgc ttg gca cta acg tgg gag aaa ctg Leu Ser Asn Ile Ala Val Ser Arg Leu Ala Leu Thr Trp Glu Lys Leu 710 715 720	2575
cca agc aag ttc aag aag ttc tat gcg gag ttt gaa agt tta atg gac Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe Glu Ser Leu Met Asp 725 730 735	2623
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act cat gag ggg aac aag acg ttc att gac aat cta gta aac ttt gaa Thr His Glu Gly Asn Lys Thr Phe Ile Asp Asn Leu Val Asn Phe Glu 775 780 785	2767
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gat gtc cgg agt tat gta cgg caa tta aat gtg att gac aac cag aga Asp Val Arg Ser Tyr Val Arg Gln Leu Asn Val Ile Asp Asn Gln Arg 820 825 830 835	2911
act tta tca cag atg tca cac aga tta gag cct cgt cga cca Thr Leu Ser Gln Met Ser His Arg Leu Glu Pro Arg Arg Pro 840 845	2953
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 35 40 45

Arg Asn Ala Ile Leu Ser Arg Ala Pro His Met Ile Arg Asp Arg Lys  
 50 55 60

Tyr His Leu Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr Glu Leu Val  
 65 70 75 80

Asp Trp Met Ile Asp Glu Thr Pro Cys Val His Ser Arg Thr Gln Ala  
 85 90 95

Val Gly Met Trp Gln Val Leu Leu Glu Asp Gly Val Leu Asn His Val  
 100 105 110

Asp Gln Glu His His Phe Gln Asp Phe Tyr Leu Phe Tyr Arg Phe Leu  
 115 120 125

Asp Asp Glu His Glu Asp Ala Pro Leu Pro Thr Glu Glu Glu Lys Lys  
 130 135 140

Glu Cys Asp Glu Glu Leu Gln Asp Thr Met Leu Leu Leu Ser Gln Met  
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Gly Pro Asp Ala His Met Arg Met Ile Leu Arg Lys Pro Pro Gly Gln  
 165 170 175

Arg Thr Val Asp Asp Leu Glu Ile Ile Tyr Glu Glu Leu Leu His Ile  
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Lys Ala Leu Ser His Leu Ser Thr Thr Val Lys Arg Glu Leu Ala Gly  
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Val Leu Ile Phe Glu Ser His Ala Lys Gly Gly Thr Val Leu Phe Asn  
 210 215 220

Gln Gly Glu Glu Gly Thr Ser Trp Tyr Ile Ile Leu Lys Gly Ser Val  
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Asn Val Val Ile Tyr Gly Lys Gly Val Val Cys Thr Leu His Glu Gly  
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Ser Ile Val Leu Arg Glu Asp Asn Cys His Phe Leu Arg Val Asp Lys  
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Glu Asp Phe Asn Arg Ile Leu Arg Asp Val Glu Ala Asn Thr Val Arg  
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Thr Val Met Ser Gly Thr Pro Glu Lys Ile Leu Glu His Phe Leu Glu  
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Thr Ile Arg Leu Glu Ala Thr Leu Asn Glu Ala Thr Asp Ser Val Leu  
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 Glu Gln Glu Lys Met Asp Tyr Ala Leu Asn Asn Lys Arg Arg Val Ile  
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 Tyr Cys Met Asp His Thr Tyr Thr Ile Arg Val Pro Val Ala Thr  
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 Ser Val Lys Glu Val Ile Ser Ala Val Ala Asp Lys Leu Gly Ser Gly  
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 Glu Gly Leu Ile Ile Val Lys Met Ser Ser Gly Gly Glu Lys Val Val  
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 Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile Cys Leu Cys  
 660 665 670  
 Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Phe Ile Lys Ile  
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 Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe Ala Ile  
 690 695 700  
 Val Met Gly Leu Ser Asn Ile Ala Val Ser Arg Leu Ala Leu Thr Trp  
 705 710 715 720  
 Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe Glu Ser  
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 Lys Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu Ile Lys Asp  
 755 760 765

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Met Thr Phe Thr His Glu Gly Asn Lys Thr Phe Ile Asp Asn Leu Val  
770 775 780

Asn Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg Thr Val Arg  
785 790 795 800

Tyr Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln Ala Asn Lys  
805 810 815

Asn His Gln Asp Val Arg Ser Tyr Val Arg Gln Leu Asn Val Ile Asp  
820 825 830

Asn Gln Arg Thr Leu Ser Gln Met Ser His Arg Leu Glu Pro Arg Arg  
835 840 845

Pro

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International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 7 :</b> C12N 15/12, C07K 14/82, 16/32, 14/47, C12Q 1/68, C12N 5/10, A01K 67/027, G01N 33/53, 33/574		<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/24768</b> <b>(43) International Publication Date:</b> 4 May 2000 (04.05.00)
<b>(21) International Application Number:</b> PCT/US99/24826 <b>(22) International Filing Date:</b> 22 October 1999 (22.10.99)		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
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<b>(74) Agent:</b> CAMACHO, Jennifer, A.; Testa, Hurwitz & Thibeault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).			
<b>(54) Title:</b> GENES INTEGRATING SIGNAL TRANSDUCTION PATHWAYS			
<b>(57) Abstract</b> <p>The present invention describes the identification, isolation, sequencing and characterization of two human CalDAG-GEF, and two human cAMP-GEF genes, which are associated with the Ras pathway. Also identified are CalDAG-GEF gene homologues in mice and cAMP-GEF gene homologues in rats. Nucleic acids and proteins comprising or derived from the CalDAG-GEFs and/or cAMP-GEFs are useful in screening and diagnosing certain Ras-associated cancers, in identifying and developing therapeutics for treatment of certain Ras-associated cancers, and in producing cell lines and transgenic animals useful as models of Ras-associated cancers.</p>			

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/24826

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12N15/12	C07K14/82	C07K16/32	C07K14/47	C12Q1/68
	C12N5/10	A01K67/027	G01N33/53	G01N33/574	

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EBINU, J.O. ET AL.: "RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs." SCIENCE, (1998 MAY 15) 280 (5366) 1082-6, XP000882708 the whole document ---	1
A	GOTOH, T. ET AL.: "Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G." MOLECULAR AND CELLULAR BIOLOGY, (1995) 15 (12) 6746-53, XP000881340 the whole document --- -/-	1

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
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- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

5 April 2000

Date of mailing of the international search report

3 July 2000 (03.07.00)

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Authorized officer

Nichogianopoulou, A

## INTERNATIONAL SEARCH REPORT

Internat:	Application No
PCT/US 99/24826	

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KAIBUCHI, K. ET AL.: "Molecular cloning of the cDNA for stimulatory GDP/GTP exchange protein fo smg p21s (ras p21-like small GTP-binding proteins) and characterization o stimulatory GDP/GTP exchange protein." MOLECULAR AND CELLULAR BIOLOGY, (1991) 11 (5) 2873-80, XP000881341 the whole document ---	1
X	KEDRA D ET AL: "THE GERMINAL CENTER KINASE GENE AND A NOVEL CDC25-LIKE GENE ARE LOCATED IN THE VICINITY OF THE PYGM GENE ON 11Q13" HUM. GENET., vol. 100, 1 October 1997 (1997-10-01), pages 611-619, XP002069545 page 613, last paragraph -page 615, paragraph FIRST; figure 2 & DATABASE EMBL [Online] AC Y12336, 19 June 1997 (1997-06-19) KEDRA D ET AL: "H. sapiens mRNA for F25B3.3 kinase like protein from C. elegans" Protein with 96.9% identity to SEQ ID No:2 and 100% identity to SEQ ID No:4 the whole document ---	1,3,5-8, 10, 38-54, 63-69
P,X	WO 98 53061 A (QUEENSLAND INST MED RES; HANCOCK JOHN (AU); SILINS GINTERS (AU)) 26 November 1998 (1998-11-26) MCG7, a hu protein with 100% identity in 609 aa overlap with SEQ ID No:4 claim 5; figure 13B ---	1,3,5-8, 10, 38-54, 63-69
P,X	KAWASAKI H ET AL: "A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia" PROC. NATL. ACAD. SCI. USA, vol. 95, October 1998 (1998-10), pages 13278-13283, XP000882748 the whole document -----	1,3,5-8, 10, 38-54, 63-69

INTERNATIONAL SEARCH REPORT

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  

Although claims 76-81, 86-109 and 127 - in as far as they concern in vivo methods - are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  

1,3-8,10,12-19,28-32,38-70,72,73-75,76,78-82,84,86,88-98,110-112,116,118,119,121-129 (all partially)

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1, 3-8, 10, 12-19, 28-32, 38-70, 72, 73-75, 76, 78-82, 84, 86, 88-98, 110-112, 116, 118, 119, 121-129 (all partially)

Claims relating to CalDAG-GEFI proteins and nucleic acids encoding them, mutants, variants, species orthologs and homologues, functional domains and antigenic determinants thereof. Methods for identifying variants or homologues of such proteins. Recombinant expression vectors, host cells and animal models for cancer expressing such recombinant modified proteins. Methods for producing said proteins and pure preparations of such. Antibodies selectively binding to such proteins, methods and cell lines for producing them. Methods for identifying compounds that modulate the expression of such proteins, and for identifying compounds that can selectively bind to them. Diagnostic methods for detecting mutations and pharmaceutical preparations comprising pure protein, expression vectors encoding the protein or antisense sequences. Pharmaceutical preparations comprising antibodies or antigenic determinants and methods of treatment.

2. Claims: 1, 3-8, 10, 12-19, 28-32, 38-70, 72, 73-75, 76, 78-82, 84, 86, 88-98, 110-112, 116, 118, 119, 121-129 (all partially)

As in subject 1, the proteins being CalDAG-GEFII proteins.

3. Claims: 2, 4-7, 9, 11, 20-27, 33-69, 71-75, 77-81, 83, 85, 87, 99-109, 113-115, 117, 118, 120-126, 130 (all partially)

As in subject 1, the proteins being cAMP-GEFI proteins.

4. Claims: 2, 4-7, 9, 11, 20-27, 33-69, 71-75, 77-81, 83, 85, 87, 99-109, 113-115, 117, 118, 120-126, 130 (all partially)

As in subject 1, the proteins being cAMP-GEFII proteins.

**INTERNATIONAL SEARCH REPORT**

International Application No	PCT/US 99/24826
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